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(71) Applicant: Wolf, Hans Joachim,
Josef-Jägerhuber-Strasse 9, D-8130 Starnberg (DE)

(72) Inventor: Wolf, Hans Joachim,
Josef-Jägerhuber-Strasse 9, D-8130 Starnberg (DE)

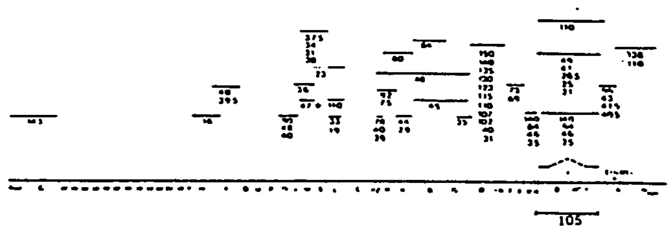
(74) Representative: Vossius Vossius Tauchner Heunemann Rauh, Siebertstrasse 4 P.O. Box 86 07 67, D-8000 München 86 (DE)

(54) **DNA sequences of the EBV genome, recombinant DNA molecules, processes for producing EBV-related antigens, diagnostic compositions and pharmaceutical compositions containing said antigens.**

(57) DNA sequences of the EBV genome, recombinant DNA molecules, processes for producing EBV-related antigens, diagnostic compositions and pharmaceutical compositions containing said antigens.

Described are DNA sequences of the EBV genome coding for EBV-related antigens, recombinant DNA molecules containing said DNA sequences, vector/host systems for cloning and expression of said DNA sequences, EBV-related antigens and methods for their preparation, diagnostic and pharmaceutical compositions containing said DNA sequences and antigens respectively (Fig. 2).

Mapping of mRNA's relative to the EBV B95-8 genome.



47* from translation in vitro correlates with p54 from in vivo labelling

VOSSIUS · VOSSIUS · TAUCHNER · HEUNEMANN · RAUH
PATENTANWÄLTE

SIEBERTSTRASSE 4 · 8000 MÜNCHEN 86 · PHONE: (089) 474075
CABLE: BENZOLPATENT MÜNCHEN · TELEX 5-29453 VOPAT D

Hans Joachim Wolf
Josef Jägerhuber Str. 9
8130 Starnberg

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DNA sequences of the EBV genome, recombinant DNA molecules, processes for producing EBV-related antigens, diagnostic compositions and pharmaceutical compositions containing said antigens

Technical field of invention

This invention relates to DNA sequences of the EBV genome coding at least for parts of EBV-related antigens to be used in methods and diagnostic and pharmaceutical compositions referred to below and methods of localising and isolating at least part of the respective DNA sequences.

Furthermore the invention relates to recombinant DNA molecules i.e. cloning and expression vectors useful for the production of antigenic determinants of said EBV-related antigens after introduction of these vectors into appropriate hosts such as bacteria, yeasts and mammalian cells.

Finally this invention relates to methods and compositions or kits, respectively, for a rapid, simple, highly sensitive and highly specific determination of antibodies directed to EBV-related antigens. In these tests different antigens of EBV are used to detect specific antibody classes in the patient's serum, directed to these antigens. This detection allows fairly reliable conclusions as to the status of infection of the serum donor such as preinfection, fresh infection, chronic infection, convalescence and neoplastic condition. Furthermore, this invention relates to pharmaceutical compositions, e.g. vaccines containing said antigens useful for prophylaxis and therapy of EBV-related diseases.

Background Art

The herpesviruses (Herpetoviridiae) are enveloped icosahedral capsids with an overall diameter of 15o nm. The viral genome consists of a double-stranded DNA with a molecular weight of approximately $1o^8$ D. Human herpesviruses are Herpes simplex I ("fever blisters"), Herpes simplex II (genital herpes), Varicella-Zoster (chickenpox, shingles), Cytomegalovirus (congenital abnormalities, e.g. microcephaly), and Epstein-Barr virus (EBV) (infectious mononucleosis (IM), Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC)

Herpesviruses display a remarkable propensity for establishing latent infections which may persist for the life of the host. After the primary infection the virus may remain quiescent, being demonstrable only sporadically or not at all, until it is reactivated by one of several known types of stimulus, such as irradiation or immunosuppression. Such exacerbations of endogenous disease may take the form of a crop of vesicles on the skin in the case of herpes simplex or zoster, or more generalized effects in the case of cytomegalovirus or EBV. The capacity to persist

indefinitely as a latent infection enables these viruses to survive in nature for a long time. During the last years, attention has turned to the correlation of human cancer and EBV.

Epstein-Barr-Virus (EBV), infections and their consequences

EBV causes infectious mononucleosis as a primary disease. Predominantly it affects children or young adults. More than 9o % of the average adult population is infected by EBV that persists for lifetime in peripheral B-lymphocytes. The virus is lifelong produced in the parotid gland and spread via the oral route.

Serology suggests that EBV might be involved in causing two neoplastic diseases of man, African Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC). Infectious mononucleosis is a consequence of primary infection by EBV. It is not a life-threatening disease if additional risk factors are absent.

However, the subjective feeling of sickness, frequently for extended periods (in the order of several weeks), and the necessity to avoid physical stress due to the drastically increased risk of spleenic rupture would certainly suggest a control of this disease.

The clinical diagnosis of infectious mononucleosis is usually derived from a combination of the following parameters:

1. High leukocyte count ranging from 1o,ooo to 2o,ooo and reaching up to 5o,ooo
2. 1o % atypical cells
3. lymphadenitis
4. fever.

Patients with infectious mononucleosis shed EBV in their saliva. Virus shedding does not require special prevention against spreading the disease as epidemics and infection of persons in close contact are rare (A.S. Evans, "The transmission of EB viral infections. Viral Infections in Oral Medicine.", edited by J. Hooks, G. Jordan, Elsevier North Holland Amsterdam, p. 211 (1982)). Virus shedding does not stop with recovery from disease and at least 60 % (possibly up to 1oo %) of the adult population shed at least low levels of EBV which is produced lifelong in epithelial cells of the salivary duct of the parotid gland (H. Wolf, M. Haus, E. Wilmes, "Persistence of Epstein-Barr virus in the parotid gland", J. Virol. 51 (1984)).

About 1 % of the infectious mononucleosis cases show complications either already at the onset of the disease or as a late consequence. Most complications are due to autoimmune mechanisms and are in some cases indiscernable from graft versus host disease, a mechanism by which the body might clear itself from the excess of EBV converted proliferating B-cells.

If the T-cell response is insufficient, e.g. due to circumstances like treatment with high doses of Cyclosporin A in combination with corticosteroids or due to AIDS or a certain genetic predisposition as described by Purtilo (Duncan's syndrome, X-chromosome-linked lymphoproliferative disease (XLP); D.T. Purtilo, K. Sakamoto, V. Barnabei, J. Seeley, T. Bechtolg, G. Rogers, J. Yetz, S. Harada and the XLP-collaborators: "Epstein-Barr virus-induced diseases in boys with the X-linked lympho-proliferative syndrome (XLP). Update on studies of the registry." Am. J. Med. 73, p. 49 (1982)), infected B-cells may have a chance to escape from host control and grow without limitation as they would do when being

cultivated in vitro. The consequences have been described as BL-like disease in cases of AIDS patients (J.L. Ziegler, R.C. Miner, E. Rosenbaum, E.T. Lennette, E. Shillitoe, C. Casavant, W.L. Drew, L. Mintz, J. Gershor, J. Greenspan, J. Beckstead, K. Yamamoto, "Outbreak of Burkitt's-like lymphoma in homosexual men.", Lancet 2, p. 631 (1982)) or as a polyclonal lympho-proliferative disease for XLP-patients (D.T. Purtilo et al., supra) or kidney transplant recipients (D.W. Hanto, G. Frizzera, D.T. Purtilo, K. Sakamoto, J.L. Sullivan, A.K. Saemundsen, G. Klein, R.L. Simmons, J.S. Najarian, "Clinical spectrum of lympho-proliferative disorders in renal transplant recipients and evidence for the role of Epstein-Barr virus.", Cancer Res. 41, p. 4253 (1981)).

The positive and fast identification of infectious mononucleosis or acute EBV infection is especially important in cases where a differential diagnosis to leukemia or, in case of transplant recipient, to graft rejection crisis is necessary. In these cases, a false diagnosis may lead to incorrect therapy, which may have serious, even life-threatening effects.

<u>Prevention of primary disease caused by EBV</u>

Infectious mononucleosis seems to be unknown in areas like the Philippines or Malaysia (D.S.K. Tan, "Absence of infectious mononucleosis among Asians in Malaya.", Med. J. Malaya 21, p. 358 (1967)) where infection by EBV occurs very early in life. Almost the whole population has antibodies at the age of 2-1o years at the latest. Clinical symptoms seem to be a consequence of juvenile or adult infection. It can be assumed that a vaccine-primed organism will be infected without significant clinical symptoms and that the consequences often fatal in the risk groups listed above could be eliminated by a vaccine.

## Burkitt's Lymphoma and EBV

The development of Burkitt's lymphoma is linked to chromosomal rearrangements. Not all cases contain EBV genomes in the tumor cells. However, at least in areas with high incidence, 97 % of these neoplasias are EBV-related and a control of EBV infection is likely to reduce the risk of developing Burkitt's lymphoma.

## Nasopharyngeal carcinoma as a possible "secondary disease" related to EBV

The other disease where EBV shows a 1oo % association is nasopharyngeal carcinoma (NPC) ("The Biology of Nasopharyngeal Carcinoma", UICC technical report series, vol. 71, edited by M.J. Simons and K. Shanmugaratnam, International Union Against Cancer, Geneva, p. 1 (1982)). NPC most frequently starts at the fossa of Rosenmueller (Recessus pharyngeus) at the postnasal space. Frequently patients are hospitalized only after the first typical metastases have developed in the cervical lymph nodes.

In some areas of Southern China and amongst Chinese in Singapore and Malaysia, NPC is the most frequent neoplasia of man with an incidence of up to 4o per 1oo,ooo per year. In other parts of the world, like Borneo or Tunesia the incidence is also high. In most other areas, the incidence is around o,2 per 1oo,ooo per year which represents about 4 % of ear, nose and throat (ENT)-tumors. The age distribution shows a clear single peak around the age of 4o to 5o in almost all high-risk areas. In Borneo and to some extent in Tunesia, a remarkable second peak has, however, been observed at an early age ranging from 5 to 15 years (M. J. Simons et al., supra).

Environmental factors including traditional Chinese medicine may be responsible for the increased risk of nasopharyngeal carcinoma in certain, predominantly Chinese, populations of Southern Asia (H. Wolf, "Biology of Epstein-Barr virus in: "Immune deficiency and cancer: Epstein-Barr virus and lymphoproliferative malignancies", ed. D. Purtilo, Plenum Press, p. 233 (1984)).

Control of EBV-related neoplasia

There are three possible basic strategies to control neoplasia:

1. Early detection followed by therapy,
2. delay of onset of disease ideally beyond the average lifespan, and
3. prevention.

These goals may be achieved also in multifactorial diseases such as many neoplasias. Incidence of disease may be reduced by eliminating one or more of the essential factors which are not necessarily sufficient by themselves to cause the disease, or by reducing factors which promote the manifestation of neoplastic conditions. The use of the specific virus-related antigens of this invention, or antibodies or genetic materials as tools for early diagnosis of virus-related tumors, might facilitate the elimination of essential factors.

Selection of EBV-related gene products for diagnosis of EBV-related NPC

A. Primary infection with EBV: Development of antibodies against VCA (viral capsid antigen), EA (early antigen) and EBNA (Epstein-Barr Nuclear Antigen)

EBV infects B-lymphocytes during acute or primary infection (mononucleosis). Due to the lack of immune response, a number of cells enter into the lytic cycle and produce a full set of viral antigens which are shed into the blood stream during cytolysis. Against these antigens, specific antibodies will be synthesized by the host's immune system (Table A).

Probably not all B-lymphocytes are capable of supporting a fully lytic infection due to a cellular factor which prevents expression of EBV. These cells are latently carrying EBV genomes for the rest of the host's life.

Table A

| DISEASE | VCA: IGG | IGM | IGA | EA | EBNA | MA[1] |
|---|---|---|---|---|---|---|
| NORMAL ADULTS | + | - | - | - | + | + |
| ACUTE ADULTS (EARLY) | ++ | + | - | + | - | - |
| CHRONIC INFECTION | + | + | - | ± | ± | ±? |
| REACTIVATION | + | + | - | + | + | + |
| XLP[2] | + | - | - | ± | (+) | ? |
| NPC | ++ | - | + | +(D) | + | ++ |
| BL | ++ | - | - | +(R) | + | + |

[2] XLP AS AN EXAMPLE OF IMMUNOLOGICALLY DEPRIVED HOSTS

[1] DETERMINED BY IMMUNOPRECIPITATION OF GP 240/200

(MA; membrane antigen)

B. Convalescence: Disappearance of antibodies against EA and maintenance of antibodies against VCA and EBNA

As the immune defense mechanisms of the body remove the

lytically infected cells from the circulation, the antibody levels will start to fall during the convalescent phase. After a certain period, anti-EA-antibodies disappear. However, as mentioned above, EBV is produced in the parotid gland. The viral particles and intracellular virus-associated antigens including EA will be shed into the saliva and reach the oropharynx. Here the viral particles bind to the B-lymphocytes and are presented to the body as antigens, thus the antibody titer against VCA is maintained. Since EA cannot bind to the lymphocytes it will be degraded by poteases and therefore will not be available to the immunesystem as an antibody-inducing antigen.

The circulating lymphocytes that are latently infected by EBV contain EBNA. At the end of their life cycle these cells disintegrate and release EBNA into the blood stream. Therefore antibodies to this antigen will persist.

Thus, due to the EBV-production in the parotid gland and to the release of EBNA from latently infected B-cells, sera of convalescents will have low anti-VCA and anti-EBNA IgG-antibody levels (see Table A, supra).
In addition EA released from rare B lymphocytes which may enter a lytic cycle may be an inferior antigen and may not give rise to antibody levels detectable with the test systems used.

In combination with the known sequence of appearance of antibody classes, specifically the early presence of IgM antibodies followed by IgG antibodies, the various antigen classes of primary disease caused by EBV can be utilized for improved diagnostic procedures. However, available test systems which are mainly based on cellular antigens or cell derived antigens have serious limitations. This concerns the sensitivity, especially for detection of IgM antibodies and also unspecific reactions.

C. EBV-related antibodies in individuals suffering of NPC:

The first suggestive evidence that Epstein-Barr virus might be causally related to nasopharyngeal carcinoma and African Burkitt's Lymphoma was derived from serological data (for review see M.A. Epstein, B.G. Achong, "The Epstein-Barr Virus" Springer Verlag Berlin, Heidelberg, New York (1979)).

Using mainly indirect immunofluorescence on cells producing virus or at least early viral antigens, significantly higher antibody titers to these antigens were found in patients' sera. These first tests which detected unspecified immunoglobulin classes against a group of proteins named Early Antigen (EA) and another group of proteins named Virus Capsid Antigens (VCA) were helpful for the establishment of a relationship between EBV and these diseases. These tests, however, are of limited value for definite diagnosis of the malignancies from a single serum, and cannot be used for monitoring therapy.

The introduction of antigen and antibody class specific tests, specifically the determination of peripheral IgA antibodies for the two antigen families EA and VCA and also the first attempts to subdivide at least the EA-family (EA, D or R; G. Henle, W. Henle and G. Klein, "Demonstration of 2 distinct components in the early antigen complex of Epstein-Barr Virus infected cells", Int. J. Cancer 8, p. 272 (1971)) achieved remarkable improvements of the diagnostic and prognostic value of the tests.

In the areas of high risk for NPC, 1% of the adult population has IgA antibodies for EBV-Capsid antigen (VCA).

Three percent of this group has NPC upon clinical examination and, with the exception of terminal cases, there were no anti-VCA IgA negative cases detected. Out of the IgA anti-VCA positives, about 1% per year developed NPC in a 3 year follow up. A test of this quality, if available as a highly specific automat- readable ELISA test, would provide an excellent "first step" screening for a population of extreme risk.

Detection of EB virus IgA/VCA antibody is helpful for diagnosis of NPC (see table on page 14), and of special value for the detection of early stages. For example, in Wuzhou City (China; high risk area for NPC), the frequency of NPC detected by serological mass survey revealed a much higher percentage of patients in stages I (42%) and II (48%) than otherwise detected in outpatient clinics (1.7% stage I and 3o % stage II). The chance of survival is clearly related to the stage at which therapy is begun. The survival rates for stage I are (according to Shanghai Tumor Hospital) 93%, for stage II 75%, and are very low for more advanced stages. Therefore it is possible to reduce the mortality rate of NPC through early detection and early treatment.

IgA antibodies to the early antigen complex of EBV can be detected in 40 % to 70 % of NPC patients, depending on the method used. These antibodies are virtually absent in the non-tumorbearing population. Such test of the tumorbearing individuals should be of great importance for the decision to start therapy, and its value would be even higher if the sensitivity could be enhanced to allow detection of disease in closer to 100 % of the tumor patients.

The detection rate of NPC among IgA/VCA antibody-positive individuals is 1.9 % and that of IgA/EA individuals is 30-40 %. These data indicate that the IgA/EA antibody

test is more specific for the detection of NPC, but not as sensitive as IgA/VCA antibody.

A number of laboratories have used the continuous determination of IgA antibodies to EA and VCA to monitor the success of therapy and for early detection of relapse with very good success.

Membrane protein gp 250/350 and its use

Four proteins of the viral envelope constituting the so-called membrane antigen complex (MA) have been described (L.F. Qualtiere, G.R. Pearson, et. al., supra; J. North, A.J. Morgan, M.A. Epstein, "Observations on the EB virus envelope and virus-determined membrane antigen (MA) polypeptides", Int. J. Cancer 26, p. 231 (1980)). Two of these proteins, i.e. gp 250 and gp 350, are antigenically closely related (D.A. Thorley-Lawson and K. Geilinger, "Monoclonal antibodies against the major glycoprotein (gp 250/350) of Epstein-Barr virus neutralize infectivity", Proc. Natl. Acad. Sci, USA 77, p. 5307 (1980)). The molecular weight of one component ranges from 200,000 to 250,000 D depending on the cell line where the virus is derived from and the second antigenetically related glycoprotein has a molecular weight of 300,000-350,000 D but is absent in some cell lines. Since these glycoproteins are all related in antigenicity, protein and encoding DNA sequence, they are usually referred to as gp 220/350 or gp 250/350 or simply as gp 250 or gp 350 but meaning the whole family of related glycoproteins.

Glycoprotein 250/350 is able to bind to the EBV receptor of human and some primate B-lymphocytes and to thus initiate the infection of these cells (A. Wells, N. Koide, G. Klein, "Two large virion envelope glycoproteins mediate Epstein-Barr virus binding to receptor-positive cells",

J. Virol. 41, p. 286 (1982)). Antibodies against these proteins neutralize the infectivity of the virus, which could be demonstrated for human as well as for rabbit antisera and mouse monoclonal antibodies (D.A. Thorley-Lawson et. al., supra). By the use of monoclonal antibodies it has been shown that blocking of only one antigenic determinant present both in gp 350 and gp 250 was sufficient for virus neutralization. Adsorption of human sera to immobilized gp 350 and gp 250 removed the neutralizing antibodies (D.A. Thorley-Lawson et. al., supra). Thus, there is convincing evidence that

a) gp 350 and gp 250 induce the production of neutralizing antibodies, and that
b) antibodies against gp 350 and gp 250 have neutralizing capacity.

Therefore, this protein as well as its related viral gene product, gp 350 (with a molecular weight of 350,000), are candidates for a possible EBV vaccine (A.J. Morgan, M.A. Epstein, J.R. North, "Comparative immunogenicity studies on Epstein-Barr virus membrane antigen (MA) gp 34o with novel adjuvants in mice, rabbits and cotton-top tamarins", J. Med. Virol. 13, p. 281 (1984)). These glycoproteins are expressed on induced EBV producer cell lines and can be easily demonstrated after radioiodination of cell surface proteins (L.F. Qualtiere, G.R. Pearson, "Epstein-Barr virus-induced membrane antigens: immunochemical characterization of Triton X-1oo solubilized viral membrane antigens from EBV superinfected Raji cells", Int. J. Cancer 23, p. 808 (1979)).

Application of gp 25o/35o for the diagnosis of EBV-related diseases

IgG antibodies are absent during the acute phase of primary EBV infection, but present for lifetime after convalescence. IgM antibodies are present in the early stage of the disease and absent during convalescence.

IgA antibodies against EBV-antigens are present almost exclusively in NPC patients and can be detected in sera of at least 58 % of these patients even with not very sensitive tests (Zeng Yi and Hans Wolf, manuscript in preparation and example 16, infra).

Comparison of Positive Rate of IgG and IgA Antibodies to VCA and MA from NPC Patients and Normal Individuals

| | Cases | MA/IgG | | MA/IgA | | VCA/IgA | | EA/IgA | |
|---|---|---|---|---|---|---|---|---|---|
| | | (+) No. | (+) rate% | (+) No. | (+) rate% | (+) No. | (+) rate% | (+) No. | (+) rate% |
| NPC Patients | 48 | 48 | 100 | 28 | 58.3 | 48 | 100 | 31 | 64.6 |
| Normal Individuals | 48 | 47 | 97.9 | 0 | 0 | 0 | 0 | 0 | 0 |

* MA/IgG and MA/IgA detected by immunofluorescence test
VCA/IgA and EA/IgA detected by immunoenzymatic test

The whole gp 25o molecule or parts of its backbone polypeptide chain can be utilized as reagents in preferentially class-specific antibody detection tests such as passive hemagglutination, counter gel electrophoresis, radioimmunoassays or enzyme-linked immuno-absorbent assays.

Highly specific test antigens allow better signals and detect otherwise unrevealed low antibody levels of clinical significance. The use of singular antigenic sites of the gp 25o instead of the entire gene product may, in some cases, permit a more precise diagnosis of the disease.

Application of gp 25o/35o for prophylaxis and treatment of EBV-related disease s

A. Since infection by EBV early in life only causes subclinical seroconversion, it may be anticipated that the presence of maternal antibodies or antibodies induced by a vaccine will influence the clinical manifestation of a primary EBV infection. It is expected that the vaccination of children or young adults, preferably before the peak of risk of catching an EBV infection, reduces effectively the clinical manifestation of infectious mononucleosis in the population.

B. In all areas with high incidence rates for NPC or BL, the population shows almost 1oo % seroconversion to EBV within the first one to two years of life. Vaccination will have to take place soon after birth. If this vaccination is regularly repeated, it will in all probability prevent EBV infection, delay it or reduce the biological effects of early primary infection. Each of these consequences is expected to either prevent the subsequent development of neoplasia, to delay its onset considerably or to decrease the relative risk.

C. In NPC, occasional production of viral antigens at the site of the tumor will stimulate primarily IgA secreting B-lymphocytes. IgA antibodies are capable of blocking antibody-mediated cytotoxicity. IgA antibodies to viral membrane antigens, such as gp 25o, are present in NPC and BL patients and may not only be indicators of the disease, but may even contribute to the failure of the immune system to eliminate the tumor cells by their masking potential. Large doses of the purified antigen given to tumor patients may bind IgA and initiate the formation of an excess of IgG antibodies directed to the same antigen. These specific IgG antibodies may then compete with remaining IgA antibodies and allow the elimination of tumor cells by antibody-dependent mechanisms.

D. Appropriate administration of gp 25o or related products might also enhance the cellular immune mechanisms and thus restrict the growth of tumors.

<u>Production of EBV specific antigens according to the present invention</u>

1. As a consequence of all findings, it is one of the objects of this invention to improve the sensitivity of tests for detection of antibody classes and antigen specific antibodies and to develop a system which allows mass testing and better standardization.

2. EBV cannot be efficiently produced in a lytic cell cycle since efficiently infectable cells are not known at present and because all of the cells used as source for the preparation of EBV or related antigens are immortalized cells or even tumor derived cells. In most cell lines retroviruses have been demonstrated. The products isolated from such cultures therefore are not only very expensive but their use is also a potential safety risk.

3. The application of recombinant DNA technology has made possible the production of useful polypeptides by appropriate host cells transformed with recombinant DNA molecules and grown in appropriate culture systems.

4. According to the present invention, recombinant DNA methods are used to express the genetic information of the genes or at least of parts of the genes encoding the EBV proteins p138, p150 and gp 250/350 in appropriate host cells, such as bacteria (e.g. the genera Escherichia, Salmonella, Pseudomonas or Bacillus), yeasts (e.g. the genera Candida, or Saccharomyces) and mammalian cells (e.g. Vero-cells, CHO-cells or lymphoblastoid cell lines).

5. Furthermore, the genomic regions encoding the EBV proteins p150, p143, p138, p110, p105, p90, p80 and p54 were identified and their relevance for diagnostic purpose has been identified. Therefore, the key information for the production of these proteins or antigenic determinants thereof in a manner as demonstrated for the proteins p138, p150 and gp 250/350 is also disclosed in the present invention.

## Recombinant DNA technology

### A. Expression control systems

Prokaryotes most frequently are represented by various strains of E. coli. However, other microbial strains may also be used, such as bacilli, for example Bacillus subtilis, various species of Pseudomonas, or other bacterial strains. In such prokaryotic systems, plasmid vectors which contain replication sites and control sequences derived from a species compatible with the host are used. For example, E. coli is typically transformed using derivatives of pBR322, a plasmid derived from an E. coli species by Bolivar, et al., Gene 2, p. 95 (1977). pBR322 contains genes for

ampicillin and tetracycline resistance, and these markers can be either retained or destroyed in constructing the desired vector. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta-lactamase (penicillinase) and lactose (lac) promoter systems (Chang, et. al. Nature 198, p. 1056 (1977)) and the tryptophan (trp) promoter system (Goeddel, et al., Nucleic Acids Res. 8, p. 4057 (1980)). The lambda-derived $P_L$ promoter and N-gene ribosome binding site (Shimatake, et al., Nature 292, p. 128 (1981), which has been made useful as a portable control cassette are further examples. However, any available promoter system compatible with prokaryotes can be used.

In addition to bacteria, eukaryotic microbes, such as yeast, may also be used as hosts. Laboratory strains of Saccharomyces cerevisiae, Baker's yeast, are most used, although a number of other strains are commonly available. While vectors employing the 2 micron origin of replication are illustrated (J.R. Broach, Meth. Enz. 101, p. 307 (1983)), other plasmid vectors suitable for yeast expression are known (see, for example, Stinchcomb et al., Nature 282, p. 39 (1979); Tschempe et al., Gene 10, p.157 (1980) and L. Clarke et al., Meth. Enz. 101, p. 300 (1983)). Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess et. al., J. Adv. Enzyme Reg. 7, p. 149 (1968); Holland et al., Biochemistry 17, p. 4900 (1978)). Additional promoters known in the art include the promoter for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255, p. 2073 (1980)), and those for other glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate iso-

merase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and enzymes responsible for maltose and galactose utilization (Holland, supra).

Evidence suggests that terminator sequences are desirable at the 3' end of the coding sequences. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes. Many of the vectors illustrated contain control sequences derived from the enolase-I gene containing plasmid peno46 (M.J. Holland et al., J. Biol. Chem. 256, p. 1385 (1981)) or the LEU 2 gene obtained from YEp13 (J. Broach et al., Gene 8, p. 121 (1979)), however, any vector containing a yeast-compatible promoter, origin of replication and other control sequences is suitable.

It is also, of course, possible to express genes encoding polypeptides in eukaryotic host cell cultures derived from multicellular organisms. See, for example, Cruz and Patterson, editors, "Tissue Cultures", Academic Press (1973). Useful host cell lines include VERO and HeLa cells, and Chinese hamster ovary (CHO) cells. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the commonly used early and late promoters from Simian Virus 40 (SV 40) (Fiers et al., Nature 273, p. 113 (1978)), or other viral promoters such as those derived from polyoma. Adenovirus 2, bovine papiloma virus, or avian sarcoma virusses. General aspects of mammalian cell host system transfor-

mations have been described by Axel in U.S. Patent No. 4,399,216 issued August 16, 1983. It now appears also that "enhancer" regions are important in optimizing expression; these are, generally, sequences found frequently upstream of the promoter region. Origins of replication may be obtained, if needed, from viral sources. However, gene integration into the chromosome is a common mechanism for DNA replication in eukaryotes, and hence independently replicating vectors are not required. Plant cells are also now available as hosts, and control sequences compatible with plant cells such as the nopaline synthase promoter and polyadenylation signal sequences (A. Depicker et al., J. Mol. Appl. Gen. 1, p. 561 (1982)) are available.

B. Transformation of suitable hosts

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by S.N. Cohen, Proc. Natl. Acad. Sci. (USA) 69, p. 2110 (1972) is used for prokaryotes or other cells which contain substantial cell wall barriers. Infection with Agrobacterium tumefaciens (C.H. Shaw et al., Gene 23, p. 315 (1983)) is used for certain plant cells. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb (Virology 52, p. 546 (1978)) is preferred. Transformations into yeast are carried out according to the method of P. van Solingen et al. (J. Bact. 130, p. 946 (1977)) and C. L. Hsiao et al. (Proc. Natl. Acad. Sci. (USA) 76, p. 3829 (1979)). Alternatively, the procedure of Klebe, et al. (Gene 25, p. 333 (1983)) can be used.

C. Construction of recombinant cloning and expression vectors

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligodeoxyribonucleotides are cleaved, tailored and religated in the form desired.

Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog.

If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in "Methods in Enzymology" 65, p. 499-560 (1980).

Restriction cleaved fragments may be blunt ended by treating with the large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs). The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only a selected one or more dNTPs within the limitations dictated by the nature of the sticky ends. Treatment under appropriate conditions with S1 nuclease results in hydrolysis of any single-stranded portion.

Synthetic oligonucleotides may be prepared by the triester method of Matteucci et al. (J. Am. Chem. Soc. 103, p. 3185 (1981)) or the diethylphosphoramidite method of Caruthers, described in U.S. Patent No. 4,415,732, issued November 15, 1983.

Ligations are performed under standard conditions and temperatures (as described below) using T4 DNA ligase. In vector constructions employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) in order to remove the 5' phosphate and prevent religation of the vector. BAP digestions are carried out under standard conditions (as described below).

D. Selection of transformants

In the constructions correct ligations for plasmid construction are confirmed by transforming E. coli or other suitable hosts with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or using other markers depending on the mode of plasmid construction, as is understood in the art.

Brief summary of the invention

The present invention relates to the production of EBV specific antigens by recombinant DNA technology and their use in diagnosis, prophylaxis and therapy of EBV-related dieseases. Therefore, it is an object of this invention to identify novel Epstein-Barr viral antigens, such as p150, p143, p138, p110, p105, p90, p80, p54 (G.J. Bayliss, H. Wolf, infra), which are correlated with Epstein-Barr virus related diseases like nasopharyngeal carcinoma (NPC), infectious mononucleosis, and Burkitt's lymphoma (see legend to Fig. 1 and Fig. 28) by immunological methods.

Another object of this invention is the localization and identification of genomic regions of EBV, for example as it has been cloned from B95-8 cells (American Type Culture Collection, Rockville, Maryland, USA (ATCC) CRL1612) (J. Skare, J.L. Strominger, "Cloning and mapping of BamHI endonuclease fragments of the DNA from the transforming B95-8 strain of Epstein-Barr Virus", Proc. Natl. Acad. Sci. USA 77, p3860(1980)) coding for said antigens of diagnostic importance and of relevance for medical purposes. This is achieved by using the hybrid selection method.

A further object of the present invention is the subcloning of a genomic region of EBV,for example from existing libraries of EBV, cloned from B95-8 cells which encodes at least a part of useful antigens for medical purposes, such as p138 and p150. This is achieved by joining a subgenomic fragment, e.g. an XhoI-fragment, derived from the EBV B95-8 subclone pBR322 BamA, (J.Skare et al., supra) to the plasmid pUC8 (J. Messing, infra) (pUC635, see Fig. 4).

Another object of this invention is the production of proteins by expression of the respective genetic information in suitable host cells, such as bacteria (e.g. of the genera Escherichia, Salmonella, Pseudomonas or Bacillus), yeasts (e.g. of the genera Candida or Saccharomyces) animal cells and human cells (e.g. Vero-cells; CHO-cells; CHO dhfr$^-$ cells in combination with an appropriate selection system, optionally a plasmid carrying a functional dhfr gene as well as the genetic information for the EBV gene under control of a suitable regulation sequence; or lymphoblastoid cell lines).The proteins produced by these host cells contain e.g. p138, p150 or gp250/350-related antigenic determinants (epitopes) and are, depending on the expression system, synthesized either as a fusion protein or as a non-fusion protein.

For the production of a fusion protein by bacteria the expression of the genomic subfragments, for example that encoding a part of p138 of EBV B95-8 and introduced into the known plasmid pUC8, was induced e.g. by isopropyl-ß-D-thioglactopyranoside (IPTG). The respective expression products were identified by immunological methods.

Another fusion protein is provided by cleaving subclone pUC635 with EcoRI and BglII and introducing this fragment into the vector plasmid pUC9 (U. Rüther, infra). The resulting recombinant plasmid is pUC924 (Fig. 6). The expression product has a size of about 94 kd.

A further fusion protein is produced by expressing the genetic information of said XhoI-p138-encoding fragment of pBR322 BamA in the plasmid pEA305 (E. Amann, J. Brosius, M. Ptashne, "Vectors bearing a hybrid trp-lac-promoter useful for regulated expression of cloned genes in E. coli", Gene 25, p. 167 (1983)). After putting the p138 related information into a proper reading frame relative to pEA305, the clone pMF924 synthezises a fusion protein that contains a part of the $\lambda$-repressor protein $c_1$ (Fig. 7).

Still another fusion protein is provided by cloning a 3.0 kb genomic XhoI-fragment containing p138-related genetic information 3' to a hybrid trp-lac promoter (as described by F. Amann et. al., supra). For this purpose the known plasmid pKK240-11 was used. The resulting clone pKK378 synthezises a fusion protein that is composed of an aminoterminal methionine residue followed by p138 related DNA sequences (Fig. 8).

Still another object of the present invention is to provide fusion proteins, non-fusion proteins or oligopeptides which contain only the antigenic determinant protein subregions of viral proteins like p138. For this purpose the determinants of the protein are located by a computer-directed analysis, using a computer program developed by

us for the Digital Equipment VAX 11/750 computer. A similar program has been used for other problems and another computer by G.H. Cohen, B. Dietzschold, M. Ponce de Leon, D. Long, E. Golub, A. Varrichio. L. Pereira, R.J. Eisenberg, "Localization and synthesis of an antigenic determinant of Herpes simplex virus glycoprotein D that stimulates the production of neutralizing antibody", J. Virol 49, p. 102 (1984). By cloning the respective fragments into vectors like pUC8 or pUR288 (U. Rüther et al., infra) plasmids as pUR600 and pUR540 were obtained. The produced large and small fusion proteins are investigated by gel electrophoresis and immunoblotting experiments. The cloning experiments in pUR288 were done for stabilizing the small p138-related polypeptides.

A further object of the present invention is the expression of polyantigens composed of antigenic determinants of several different EBV-serotypes. For that purpose the corresponding DNA fragments are linked and introduced in a suitable vector. The expression products are fusion and non-fusion EBV-specific polyantigens.

Further fusion proteins containing p150-related antigenic determinants were obtained by cloning and expression of the corresponding DNA sequences in pUR plasmids and pUC plasmids. The obtained constructs were the recombinant plasmids pUR290CXH580, pUR290DBX320, pUR292DBB180, pUR290DTT700, pURDTT740, pUR290DTP680, and pUR288DPP320.

Another object of the present invention is the construction of new expression vectors, such as pUC600 and pUC601 which contain a part of the coding region of the viral protein p138. If DNA sequences are introduced 3' to this sequence into the vector, the expression products are stabilized by the p138-specific aminoacid sequence and protected against protease degradation.

Still another object of this invention is the modification of said expression vectors by introducing a DNA-sequence coding for three to fifteen Arginine residues and at least one stop codon 3' of the cloning site of said expression vectors and furthermore positioned in an appropriate reading frame. The obtained vector is pUCARG601. If DNA-sequences coding for proteinaceous material are inserted into this expression vector the expression products will be fusion proteins carrying said Arginine residues at their carboxy terminus, such as those fusion proteins encoded by plasmids pUCARG1140 (see Fig. 12a)) and pUCARG680.

Thus it is an object of this invention to provide a simple method for isolating proteins useful for diagnosis, prophylaxis and therapy such as EBV p138 or related polypeptides or oligopeptides (antigens) from the host cell lysate according to the method of H. M. Sassenfeld, S.J. Brewer ("A polypeptide fusion designed for the purification of recombinant proteins", Bio/Technology 2, p. 76 (1984)). By introduction of said Arginine residues the net charge of the expressed proteins becomes more positive and after lysis of the host cells the oligo-arginine linked proteins are isolated by a SP Sephadex C-25 column chromatography. Due to the oligo-arginine group the EBV specific proteins are eluted at a high NaCl-concentration. This eluate is then treated with carboxypeptidase B which degrades carboxy-terminal lysine and arginine residues. Finally another SP Sephadex C-25 chromatography is carried out wherein the EBV-related proteins are eluted at low salt concentrations (see Fig.16). It is evident, however, that this procedure may be used also for the purification of proteins secreted into the medium.

It is also evident that other established methods for protein-purification such as molecular sieving or affinity chromatography on ion exchange columns or columns loaded with specific antibodies to the expressed proteins can be used as additional or alternate purification methods. For the production of non-fusion proteins which essentially contain amino acid sequences of the naturally occurring proteins or parts thereof the recombinant plasmids of the present invention may be modified. If an oligonucleotide linker is inserted between the bacterial protein encoding region and the EBV-related protein encoding region of the expression vector, the amino acid sequence corresponding to the oligonucleotide linker becomes part ot the expressed fusion protein. After isolation of this fusion protein from the transformants expressing it, it is cleaved either by amino acid sequence specific proteases in the introduced aminoacid linker or, if the amino acid linker comprises peptide bonds sensitive to acid cleavage, by treatment with acids, e.g. formic acid.

A further object of this invention is the cloning of a genomic region of EBV coding for at least a part of the specific viral antigen gp 250 and gp 350. This is achieved by joining a subgenomic PstI-PstI fragment of the EBV genome from the cell strain B95-8 (ATCC CRL 1612) (R. Baer et al., infra) contained in pBR322 BamL (J. Skare et al, supra) to the plasmid pUC8 (J. Messing et al., infra). The resulting recombinant plasmid is designated as pUCLP1.9 (see Fig. 19).

For the production of a fusion protein by bacteria, a genomic subfragment coding for a part of gp 250 and gp 350 of EBV B95-8 was cloned into the vector pUR 290 (U. Rüther et al., infra) which carries a region of the lacZ gene coding for the enzyme ß-galactosidase (pURLP1.9, see Fig. 20). The respective expression product was purified and identified by immunological methods.

Still another object of the present invention is to provide fusion proteins or non-fusion proteins which contain only the antigenic determinant protein subregions of gp 250 and gp 350. For this purpose the antigenic determinants of the proteins were localized by a computer-directed analysis using a computer program developed by us for the Digital Equipment VAX 11/750 computer.

The respective DNA-fragments are then cloned in a conventional expression vector such as pUR (ß-galactosidase) (U. Rüther, et al., infra). Plasmids obtained were e.g. pURLEP600 and pURLXP390 (see Fig. 27). Furthermore, the N-terminal antigenic determinant of gp250/350 was expressed as a fusion protein in a pUC vector (pUCLEP600, see Fig. 27). Another fusion protein is provided by cloning a DNA-fragment coding for the N-terminal antigenic determinant of gp 250 and gp 350 into the expression vector pUCARG601 mentioned above.

A further object of the present invention is the expression of polyantigens containing several antigenic determinants of gp 250 and gp 350 located by said computer analysis. For this purpose the corresponding DNA fragments are linked and introduced in a suitable vector. The expression products are fusion and non-fusion EBV-specific polyantigens.

A final object of the present invention is the utilization of either said EBV-related proteins or subregions thereof or, if suitable, EBV-related DNA fragments or clones, for the production of diagnostic compositions (kits) useful in clinical diagnosis or scientific research. These tests are based on principles as ELISA (Enzyme-linked immuno sorbent assay), RIA (Radio immuno assay) or the indirect hemagglutination assay. Furthermore, the EBV-

related proteins can be used, e.g. for monitoring vaccination programs, analyzing epidemiological problems, for patients treatment, and for the production of vaccines for prophylaxis and therapy of EBV-related diseases, such as mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma. Vaccines can be manufactured according to conventional methods. Unit doses are filled in vials optionally together with a conventional adjuvant such as aluminium hydroxide. Alternatively the product may be administered in the form of aggregates with liposomes. Patients may be vaccinated with a dose sufficient to stimulate antibody formation and revaccinated after one month and after 6 months.

Finally the proteins are useful for prophylaxis and therapy of EBV-related diseases, because they are able to modulate the immune response in patients suffering from diseases such as NPC, chronic infectious mononucleosis or EBV-related Burkitt's lymphoma.

## Brief description of the drawings

Figure 1: Autoradiography of an immunoprecipitation of EBV-specific sera derived from patients suffering from mononucleosis and NPC.

The immunoprecipitated $^{35}$S-labelled proteins were separated by a SDS-polyacrylamide gel electrophoresis and an X-ray-film was exposed to the gel.

The sources of the different sera used for precipitation are given at the bottom of the respective regions of the autoradiography. The control, designated "pool", contains all of the immunoprecipitatable EBV-specific proteins.

It can be taken from the autoradiography that at least antibodies to p138, p105 and p80 are present in each of the NPC sera and only in some of the other EBV-infection specific sera. In analogy, antibodies to p54 are significant for fresh EBV infection (infectious mononucleosis) as compared to convalescent state. Antibodies to p15o, p143, p11o, p90 are also present in convalescent sera of healthy individuals and can serve as markers for immunity or, in connection with IgM specific tests, for fresh EBV infection or, in connection with IgA, for a specific test for EBV-related neoplasia (NPC and BL).

Figure 2: Mapping of mRNA's relative to the EBV B95-8 genome.

The BamHI restriction sites of the EBV B95-8 genome are given at the bottom of the figure and the respective restriction fragments are designated by upper and lower case letters. The mRNA's of the proteins localized by hybrid-selection to individual BamHI restriction fragments are indicated by numbers and lines.
It can be taken from the figure, that the gene of p138 was correlated to the BamA-fragment.

Figure 3: DNA sequence of the leftward reading frame of BamA encoding p138.

The sequence shown is the respective negative strand. The p138 encoding region starts at nucleotide position 182 and ends at nucleotide position 3565. Restriction sites used for cloning of fragments of this coding region are indicated.

Figure 4: Restriction map of the plasmids pUC635 and pUC613o.

The size of the vector pUC8 is 2.7 kb. The cloning site 3' of the lacUV5 ß-galactosidase promoter and operator (PO) contains EcoRI(E), BamHI(B), SalI(S), PstI(P), and HindIII(H) site. The ß-lactamase gene is indicated by AMP. The 3.o kb and 3.3 kb XhoI-fragments of the p138 coding region are inserted into the SalI site of pUC8. The insertion is indicated by an open bar. pUC635 contains the 3.o kb XhoI-fragment in a correct reading frame relative to the ß-galactosidase gene,whereas pUC613o contains the 3.3 kb XhoI-fragment in the opposite orientation.

Figure 5: Expression of the proteins encoded by plasmids pUC635, pUC924, pMF924, and pKK378.

Lane 1 :of the immunostained Western-blot shows the proteins isolated from bacteria transformed with pUC8 and induced with IPTG.

Lane 2: proteins of pUC924 transformed bacteria
Lane 3: proteins of pKK378 transformed bacteria
Lane 4: proteins of pMF924 transformed bacteria
Lane 5: proteins of pUC635 transformed bacteria
The size of the fusion protein was estimated to be 75kD (lane 2), 110 kD (lane 3), 90 kD (lane 4) and 135 kD (lane 5).

Figure 6: Restriction map of the plasmid pUC924.

The size of the vector pUC9 is 2.7 kb. The cloning site 3' of the lacUV5 ß-galactosidase promoter and operator (PO) contains an EcoRI(E), BamHI(B), SalI(S), PstI(P), and HindIII(H) site. The ß-lactamase gene is indicated by AMP.

The 2.6 kb BglII/EcoRI-fragment of pUC635 is inserted between the BamHI and EcoRI sites. The abbreviation of BglII is "Bg".

Figure 7:Restriction map of the plasmid pMF924.

The 2.6 kb BamHI/HindIII-fragment of pUC924 was inserted into the BamHI and HindIII restriction sites of pEA3o5 which are located 3' of the hybrid trp-lac promoter (tac) and the aminoterminal coding region of $c_1$ ($\lambda$-repressor).

Figure 8: Restriction map of the plasmid pKK378.

The 3.3 kb BamHI/HindIII-fragment of pUC613o was inserted into the HindIII-site of the vector pKK24o-11 using a 345 bp BamHI/HindIII-fragment of pBR322 as a linker (which is indicated by a heavy black line). Thus the p138 encoding fragment is located 3' of the hybrid trp-lac promoter (tac) and an ATG start codon.

Figure 9: Secondary structures of p138.

Computer plot of Chou-Fasman calculation of the p138 secondary structure. Additionally, the hydrophobic (closed circles) and hydrophilic (open circles) regions are indicated.

Antigenic sites can be expected in hydrophilic regions with a ß-turn. This situation is given in the p6oo region and at the carboxy-terminus of the protein.

The regions subcloned into the vectors pUC8 and pUR288 are indicated.

Figure 10: Expression products of bacteria transformed with the plasmid pUR carrying PstI fragments of p138.

A. A coomassie brilliant blue stained SDS polyacrylamide slab gel analysis of lysates of IPTG induced bacteria carrying the various plasmids is shown. Fusion proteins with molecular weights between 120 and 150 kd are indicated with a closed circle. Track M molecular weight markers tracks pUR400-pUR540 lysates of bacteria carrying plasmids containing the regions of p138 as shown in Fig. 3.

B. An enzyme-linked immunoassay of proteins transfered from a gel (similar to that shown in panel A) onto nitrocellulose paper (Western blot) is shown. In this assay a pool of high titered antiserum was used and after washing, the bound immunoglobulins were visualized by sequential reaction with peroxidase coupled to antibodies against human IgG and diaminobenzidine. Only fusion proteins from bacteria containing pUR600 and pUR540 show specific reactions. Plasmid pUC635 (as a positive control) contains almost the whole of p138 coding region, however the protein is unstable and is rapidly degraded. pUC8 is the negative control containing the vector plasmid free from EBV derived sequences.

Figure 11: Expression product of bacteria transformed with the pUC subclones carrying PstI-fragments of p138.

An enzyme-linked immunoassay of proteins electrophoretically transfered from a gel onto nitrocellulose paper (Western blot) was carried out. In this assay a pool of high titered antiserum was used and after washing, the bound immunoglobulins were visualized by sequential reaction with peroxidase coupled to antibodies against human IgG and diaminobenzidine. The fusion protein from bacteria containing pUCP600 was stably produced and shows a specific antigenic reaction.

Figure 12: Construction scheme for plasmid pUCARG1140 encoding both antigenic sites found by expression as ß-gal fusion proteins

a) The 5'-PstI site in pUC600 was removed by digesting with SstI (20bp upstream) and HindIII followed by the ligation with pUC12-SstI/HindIII. From this plasmid the insert was removed with EcoRI and PstI and ligated into pUC8-EcoRI. An oligonucleotide coding for five arginines and two stop codons was inserted into the resulting plasmid pUC601 as single-stranded DNA between the 3'-PstI site and HindIII (pUCARG601). In a last step the 540bp PstI fragment encoding the second antigenic determinant from the C-terminus of p138 was inserted by digestion with PstI and ligation. The resulting plasmid contained both antigenic sites in frame followed by five arginine-residues. It was designated as pUCARG1140.

b) Nucleotide sequence of the oligoarginine linker. The lower strand was synthesized and inserted as a single-strand DNA via bridge formation between the sticky ends of PstI and HindIII.

Figure 13: IPTG-induced expression of the plasmids pUC600, pUC601, pUCARG601 and pUCARG1140 with pUC8 as a control

The upper part shows a Coomassie-stained SDS-PAGE. The newly detected proteins are marked by a black dot. The lower part shows the corresponding western blot obtained after immunostaining with serum from NPC patients. In comparison to pUCP600 the EBV-related protein encoded by pUC601 is about 1.5kD smaller due to the lack of 14 aminoacids (6 aminoacids encoded by the pUC-polylinker and 8 from the PstI-SstI fragment). The size of the protein encoded by pUCARG601 is further reduced for about 11kD since the read through into the lacZ region of pUC is inhibited by stop codons present in the inserted oligonucleotide. In pUCARG1140 the size increases to about 42 kD due to the insertion of the 540bp fragment. The protein is stable in bacterial cells.

Figure 14: Distribution and reactivity of the IgG and IgA antibodies of individual NPC-sera against the two epitopes detected in p138.

Lysates of IPTG-induced E.coli cells carrying the indicated plasmids were independently separated on a 12% SDS-PAGE four-times and the proteins were transferred to Nitrocellulose by Western-blotting. Lanes 1: pUR288 as negative control; lanes 2: pUCARG1140 as a positive control; lanes 3: pUR540; lanes 4: pUR600. Two individual NPC-sera (no. 352 and 354) were incubated with the filters and the bound IgG and IgA antibodies were visualized using peroxidase conjugated anti-human IgG and anti-human IgA rabbit antibodies. The different locations of the proteins in the Western blots, especially of pUCARG1140, result from different electrophoresis times of the SDS-PAGEs.
Whereas in NPC-serum no.352 the main reaction of the IgG and IgA antibodies is directed against the P540 epitope from the C-terminus of p138 (see Fig. 9) in serum no.354 the main part of the anti-p138 antibodies recognizes the P600 epitope (see Fig. 9). This indicates that both antigenic sites are necessary for detecting anti-p138 antibodies in sera.

Figure 15: ELISA test using the protein encoded by plasmid pUCARG1140 as antigen.
Row 1 and 3: EBV-negative sera, row 2: NPC pool serum, row 4-13: individual NPC sera. The dilutions tested are indicated at the bottom; left lane: IgG right lane:IgA.

Figure 16: Purification of proteins carrying oligo-arginine groups at their carboxy-terminus.

A. Sequence of the oligonucleotide encoding five argine residues and two stop codons. A HindIII-site at the 5'-end and a PstI-site at the 3'-end were generated for the insertion of the oligonucleotide into pUC8.

B. Purification scheme of insoluble expressed eukaryotic proteins carrying said Arg-linker at their carboxy-terminus.

Fig. 17:

DNA sequence of the leftward reading frame of the Bam L-fragment encoding gp 250/350.

The coding region for the glycoprotein starts at genomic position 92153 and ends at position 89433. The sequence shown is the respective negative strand, beginning with the BamHI site at position 92703. According to the sequence numbering in this figure the gp 350 encoding region is located between position 556 and 3276. A TATAA-box in the region of basepair 520 is marked with ..., the probable poly-adenylation site at position 3290 with +++. The splice donor and splice acceptor sites are indicated by )(--- for donor and ---)( for the acceptor site. A hydrophobic region near the carboxy-terminus of the coding region is marked with ***. Probably this aminoacid sequence serves as an anchor sequence for fixing the protein to the membrane.

Fig. 18:

Restriction map and open reading frames of the Bam L-fragment

A. Restriction map:
The positions of the restriction enzymes Bam HI, EcoRI, HindIII and PstI are indicated relative to the nucleotide positions of the Bam L-fragment.

B. Open reading frames:
The open reading frames of the Bam L-fragment are indicated as boxes and given for both polarities of the respective DNA sequence.

Fig. 19:

Restriction map of the plasmid pUCLP1.9

The size of pUC8 is 2.7 kb. The cloning region 3' of the LacUV5 ß-galactosidase promoter and operator (PO) contains an EcoRI(E), BamHI(B), SalI(S), PstI(P), and HindIII(H) site. The 1.9 kb subfragment of the Bam L-fragment, indicated by an open bar, was inserted into the PstI site. The reading frame has the same orientation as the lacZ-coding part of pUC8 (indicated by a heavy black line).

Fig. 20:

Restriction map of plasmid pURLP1.9

The vector pUR290 has a length of 5.2 kb and consists of the ß-lactamase gene ($AMP^R$) and the origin of replication of pBR322. The ß-galactosidase gene is indicated by a heavy black line, the respective promoter-operator region by PO. The restriction enzymes are abbreviated as follows: BamHI(B), ClaI(C), EcoRI(E), HindIII(H), PstI(P), and SalI(S).

The 1.9 kb insert of pUCLP1.9 was introduced between the BamHI and the HindIII site.

Fig. 21:

DNA- and amino acid sequence of the fusion protein encoded by plasmid pURLP1.9

| | |
|---|---|
| bp 4 - 3069: | ß-galactosidase |
| bp 3070 - 3072: | pUR290 linker (given in low letters) |
| bp 3073 - 3088: | pUC8 multiple cloning site (BamHI to PstI; given in low letters) |
| bp 3089 - 4985: | PstI fragment of gp 350 |
| bp 4986 - 4994: | pUC8 multiple cloning site (PstI to HindIII; given in low letters) |
| bp 4995 - END: | pBR322 sequence. |

Fig. 22:

Expression of the ß-gal : gp 350 protein encoded by plasmid pURLP1.9

Lane 1 and 2 show a coomassie blue stained PAGE of an uninduced (lane 1) and an IPTG induced (lane 2) pURLP1.9 containing clone.

Since there are a lot of bands with different molecular weights, it seems that the main part of the protein is incompletely synthesized.

Lane 3 shows a peroxidase-DAB stained Western blot with NPC sera. It is demonstrated that all newly expressed proteins are antigenic, except that band according to the size of 116 kD which corresponds to the ß-galactosidase.

The bacterial background bands are due to the high content of antibacterial-antibodies in the serum used.

Fig. 23:

Purification of the ß-gal : gp 350 fusion protein encoded by plasmid pURLP1.9

A. Coomassie stained gel; B. Western blot, treated with NPC serum

Lane 1: Uninduced culture

Lane 2: IPTG induced culture

Lane 3: Insoluble proteins of the lysed bacteria, disolved in 8M urea

Lane 4: ß-gal : gp 350 protein containing fractions, pooled after Sepharose 2B-Cl chromatography.

Figure 24: Computer-predicted secondary structure of gp350 comprising the relative values of hydrophilicity (dark circles) and hydrophobicity (grey circles). In the scale given only the loop structures can be seen clearly as line turns of 180°.

Figure 25: Expression of gp350-fragments as ß-gal fusion proteins.

The coomassie blue stained expression products encoded by plasmids pURLEP600 and pURLXP390 are shown in the upper part (pUR288 as control). In the lower part the same probes are shown after immunostaining for demonstrating their reactivity with EBV-positive sera.

Figure 26: Expression of the proteins encoded by pUCLEP600 and pUCARG1230 and their reactivity against EBV-positive sera with pUC8 as control; upper part: comassie-stained SDS-PAGE, lower part immunostained westernblot

Figure 27: Restriction map of the region coding for gp 250/350

The dark bar indicates the region coding for gp 250/350. Furthermore the restriction enzymes used for subcloning, the splice sites, and the inserts of the recombinant expression plasmids constructed according to examples 13 and 15-17 are shown.

Figure 28: DNA sequence and corresponding aminoacid sequence of EBV-related proteins.

A. Protein p54
   Nucleotide sequence and derived aminoacid sequence of protein p54 which is identified in in vitro translation as p47 but correlated with immuno-precipitation with monoclonal antibodies
B. Protein p90
C. Protein p143
D. Protein p150

Figure 29: Expression of the ß-gal::p150 fusion proteins

IPTG-induced clones indicated on top were separated after lysis in an 10 % SDS-PAGE and the proteins were stained with Coomassie-blue. As a control pUR288 was applied to show the size of the ß-galactosidase. All clones produce new proteins larger than the control clone and correspon-ding to the insert size.

Figure 30: Antigenicity of the ß-gal::p150 fusion proteins

The same lysates from clones shown in Fig. 29 were trans-ferred to nitrocellulose and EBV-related antigens were visualized by immuno staining (see supra). The clone en-coding the N-terminal part reacts strongly.

Figure 31: Map of the p150 encoding region

The p150 encoding region is shown as dark bar. The re-stricion sites used for subcloning and the resulting pUR-clones are also indicated.

Best mode of carrying out the invention

Example 1

Identification of an antigen suitable for diagnosis of NPC

In order to obtain the desired DNA sequences coding for EBV-related antigens of diagnostic significance the following strategy was developed:

Immunoprecipitation of Epstein-Barr viral proteins with various sera from normal adults, patients with fresh infectious mononucleosis or nasopharyngeal carcinoma was used to identify antigens, which are of relevance for the diagnosis of immune status and characteristic for a particular disease (Figure 1). These antigens have been localized on the Epstein-Barr virus genome by hybrid-selected translation. With the use of sequence data, these genes were subcloned from EBV-DNA and expressed in eucaryotic and procaryotic cells.

It was shown by immunoprecipitation that EA and VCA are not single antigens but families of antigens that consist of several polypeptides (G.J. Bayliss, H. Wolf, "The regulated expression of Epstein-Barr virus. III. Proteins specified by EBV during the lytic cycle", J. Gen. Virol 56, p. 1o5 (1981)).

For the immunoprecipitation the EBV-producing, MA-positive cell line P3HR1, the EBV-positive, non-producing Raji cell line and the EBV-negative cell line BJAB were used. When the cells reached a density of about $1o^6$/ml, they were diluted with an equal volume of fresh medium. For induction of EBV antigens, P3HR1 cultures were treated with 4o ng/ml phorbol-12-mystrate-13-acetate (modified from zur Hausen et al (H. zur Hausen, F.J. O'Neill, U.K. Freese, E. Hecker, "Persisting oncogenic herpes virus induced by the tumor promotor TPA", Nature 272, p. 373 (1978)) and 3 mM butyric acid immediately after subculture. For the labelling of the proteins, the cells were collected by low-speed centrifugation and resuspended at a density of $2 \times 1o^6$ cells/ml

in methionine-free MEM culture medium containing between 5o and 1oo µCi/ml $^{35}S$-methionine. The cells were incubated at 37°C/5 % $CO_2$ for 4 h and subsequently washed with cold Hanks' phosphate buffered saline (PBS) and resuspended in cold IP buffer (1% Triton-X-1oo, o.1% SDS; o.137 M NaCl; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 1o% glycerol; 2o mM Tris-HCl pH 9.o; o,o1 % $NaN_3$; 1µg/ml phenylmethylsulphonyl fluoride) at a concentration of 5 x $1o^6$ cell/ml. Then the cells were disrupted by sonication and incubated on ice for 6o min. The extracts clarified by centrifugation at 1oo,ooo x g for 3o min at 4°C.

$^{35}S$-methionine labelled extracts were immuno-precipitated exactly as described (G.J. Bayliss, et al., supra). The results are shown in Fig. 1.

Antibodies to p138, p1o5, p90 and p80 are present in each of the NPC sera and only in some of the other EBV-infection specific sera. In analogy antibodies to p54 (identical to p58 in G.J. Bayliss et al., supra) are significant for fresh EBV-infection (infectious mononucleosis) as compared to convalescent state. Antibodies to p150, p143, p110 are also present in convalescent sera of healthy individuals and can serve as markers for immunity or, in connection with IgM specific tests for fresh EBV-infection or, in connection with IgA specific tests, for EBV-related neoplasia (NPC and BL).

The next step was to localize the antigens on the EBV genome. Therefore RNA was prepared by lysing the EBV-producing cells described above with 4 M guanidine isothiocyanate and o.5 M 2-mercaptoethanol two days after induction (J.M. Chirgwin, A.E. Przybyla, R.J. MacDonald, W.J. Rutter, "Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease", Biochemistry 18, p. 5294, (1979)). The lysate was centrifuged for one

hour at 20.000 rpm (SW 41, Beckmann) and the supernatant layered on top of 2 ml CsCl density 1.8 g/cm$^3$. After centrifugation for 17 hours at 150.000 g, the RNA pellet was extracted with chloroform and precipitated with ethanol. 100 µg total cellular RNA was hybridized for 2.5 hours at 52°C in 65 % formamide and 0.4 M NaCl to 16 µg cloned EBV-DNA, which was sonicated, denatured and spotted on small nitrocellulose filters. Bound mRNA was eluted by boiling the filters 90 sec in water. The RNA was translated in vitro with a mRNA dependent rabbit reticulocyte lysate. The translation products were immunoprecipitated using 5 µl of a pool of human NPC sera for one assay after preincubation with a protein extract from unlabelled EBV-negative BJA-B cells as previously described (G.J. Bayliss, G. Deby, H. Wolf, "An immunoprecipitation blocking assay for the analysis of EBV induced antigens", J. Virol. Methods 7, p. 229 (1983)). The immune complexes were bound on protein A-sepharose, washed, eluted by boiling the beads in electrophoresis sample buffer and loaded onto SDS-polyarcrylamid gels. This procedure allowed mapping of a number of viral proteins (Fig. 2) relative to the EBV B95-8 genome. The localization of p138, is given in Fig. 2. Using sequence data (R. Baer, A.T. Bankier, M.D. Biggin, P.L.Deininger, P.J. Fawell, T. J. Gibson, G. Hatfull, G.S. Hudson, S.C. Satchwell, C. Seguin, P.S. Tuffuel, B. Barrell, "DNA-sequence and expression of the B95-8 Epstein-Barr virus genome", Nature 31o, p. 2o7 (1984)), appropriate open reading frames for p138 and p54 were identified (Fig. 2). These open reading frames are completely contained in the right part of the BamA-fragment at the right end of the viral genome.

Example 2

Cloning of the p138 encoding region

According to the sequence data of R. Baer et al., (supra), there is a large open reading frame contained in the BamA-fragment of EBV B95-8 which is suitable for encoding p138. The nucleotide sequence, the corresponding aminoacid sequence and the respective regulatory elements of the gene of p138 are given in Fig. 3.

50 µg DNA of the plasmid pBR322-BamA (J.Skare et al., supra) were digested with 50 U XhoI (Boehringer) for 2 h at 37°C in a total volume of 15o µl containing 15o mM NaCl, 1o mM $MgCl_2$, 6 mM mercaptoethanol, 6 mM Tris-HCl, pH 7.9. 3o µl stop buffer (1o mM Tris-HCl, 5o mM EDTA, 6o % sucrose, 1 % bromphenolblue, pH 7.5) were added, the mixture was put onto a preparative 1 % agarose gel in acetate-buffer (o.o4 M Tris-acetate, 2 mM EDTA, pH 7.6), and electrophoresed for 16 h with 4o V at 4°C. As a size marker HindIII digested λ-phage DNA (Boehringer) was used. After staining the gel in Tris-acetate buffer with ethidium bromide (0.5 µg/ml) for 1 h at room temperature (RT), the DNA was visualized by UV-illumination and the bands corresponding to 3.o and 3.3 kb were excised (the 3.o kb XhoI-generated fragment is the desired fragment, the 3.3 kb XhoI-generated fragment is a partial digest product (one XhoI restriction site was not cut)).

The DNA of the bands was eluted by putting the agarose pieces into dialysis bags, adding 3 volumes of Tris-acetate buffer and electrophoresed for 4 h (1oo V, 4°C). Further purification was carried out by a chromatography with Elutip D columns (Schleicher & Schuell) according to the procedure recommended by the manufacturer, extraction of the contained ethidium bromide with isoamylalcohol and pre-

cipitation of the DNA by adding 2.5 volumes ethanol and incubating overnight at -2o°C. The DNA was collected by centrifugation in a Sorvall SS 34 rotor (17.ooo rpm, 2o min) and washed with 7o % ethanol. After lyophilization the DNA was dissolved in 15 µl TE buffer (1o mM Tris-HCl, 1 mM EDTA, pH 7.5).

The DNA concentration of the two isolated fragments was estimated by electrophoresing 1 µl each in parallel with 1oo ng and 1 µg of pUC8 DNA.

SalI digested DNA of the vector pUC8 (deposited with the Deutsche Sammlung für Mikroorganismen (DSM), Göttingen , West Germany, under the accession number DSM 3420 ) (J. Messing, J. Vieira, of double-digest restriction fragments", Gene 19, p. 269 (1982)) was prepared as described before, except that for inhibition of religation of the vector during the following ligase reaction the DNA was treated with alkaline phosphatase (o.5 units (Boehringer), 3o min at 37°C).

In the following, the two purified fragments were each inserted into the cleaved vector (SalI and XhoI produce the same cohesive ends, i.e. -TGCA-). For this purpose for each of the fragments a ligation reaction was carried out with 3oo ng fragment DNA and 1oo ng pUC8 DNA in a total volume of 2o µl ligase buffer (1o mM Tris, 1o mM $MgCl_2$, 6 mM mercaptoethanol, o.6 mM ATP, pH 7.5) containing 1U T4-DNA ligase (Boehringer). After 2o h at 14°C, 80 µl TE buffer and 200 µl competent E.coli JM83 cells (ATCC 35607) (J. Vieira, J. Messing, "The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers", Gene 19, p. 259 (1982)) were added. The transformation was done according to the calcium chloride procedure (M. Mandel, A. Higa, "Calcium dependent bacteriophage DNA infection", J. Mol. Biol. 53, p. 154 (197o)). Then the cells were mixed with 1.5 ml L-broth

(5 g yeast extract, 1o g tryptone, 5 g NaCl) incubated 1.5 h at 37°C, and finally plated on L-broth agar-plates (1.5 %) supplemented with 5o µg/ml Ampicillin (Sigma) and 4o µg/ml X-gal (Boehringer). During this incubation bacteria carrying religated pUC8 molecules yield blue colonies and those which carry recombinant plasmids yield white colonies.

For identification of clones that carry the desired recombinant plasmid, twelve white colonies were picked and grown overnight at 37°C in L-broth. Aliquots of DNA-preparations according to H.C. Birnboim and J. Doly ("A rapid alkaline extraction procedure for screening recombinant plasmid DNA", Nucl. Acids Res. 7, p. 1513 (1979)) were digested by BamHI and HindIII and electrophoresed on an agarose gel as described before. Furthermore, for demonstrating the orientation of the integrated fragment, a digest was carried out with BamHI and BglII. Finally the 3.3 kb was checked by a XhoI digest.

Plasmid pUC635 carries the 3.0 kb XhoI-subfragment of the BamA-fragment (pBR322 BamA) in the proper orientation and the proper reading frame relative to the lac UV5 promoter and is used for the expression of nearly the whole p 138 (Fig. 4). The fusion protein encoded by pUC635 is composed of 12 amino acids of the ß-galactosidase amino terminus, about 1o2o amino acids of p138, 6o amino acids of the carboxy terminal part of the ß-galactosidase and another 29 amino acids of a pBR322 encoded region. Plasmid pUC6130 carries the 3.3 kb fragment in the opposite orientation (Fig. 4) Since the strain E.coli K12 JM83 is not a ß-galactosidase repressor overproducer, the fusion protein is constitutively expressed. Therefore the plasmid pUC635 was introduced into the ß-glactosidase repressor overproducer strain E.coli K12 BMH71-18 (DSM 3413) (U. Rüther, B. Müller-Hill, "Easy identification of cDNA clones", EMBO Journal 1o, p. 1791

(1983)). Instead of strain E. coli K12 MBH71-18 strain E.coli K12 JM109 (DSM 3423) can also be used (without essential alteration of the experimental procedure).

Besides pUC635 three other plasmids were constructed: pUC924, pMF924 and pKK378 (Figures 6 to 8).

The insert of pKK378 starts at the same XhoI-site and continues up to the third XhoI-site located 250bp 3' of the stop codon. This fragment of 3.3kb was generated by an incomplete digest and inserted behind the tac-promotor and the start codon of pKK240-11 (F.Amann et al., supra). The expression product contains only two bacterial amino acids and its size is smaller then the size of the expression product of pUC635 because the bacterial lacZ part is missing.

pUC924 contains the fragment from the Bgl II-site to the third Xho I site. pUC9 (DSM 3421) was used as vector. Since the size of the insert is smaller than in pUC635 and since the stop codon from p138 is used, the molecular weight of the expression product is expected to be smaller than in pUC635 and pKK378.

The plasmid pMF924 was constructed from pEA305 (E. Amann et al., supra) and the same BglII-XhoI fragment as in pUC924. pEA305 has a tac-promotor followed by the N-terminal part of the C1repressor, the resulting fusion protein is expected to be 17kd larger than in pUC924.

These constructs were tested for the production of EBV-related antigens by inducing the tac- and lac-promotors with IPTG and separating the proteins on an SDS-PAGE. None or only weak new bands could be detected on Coomassie-blue stained gels in the regions with the expected sizes. But after a transfer of the proteins onto nitrocellulose and immunostaining with a high titered NPC-pool serum and a peroxidase conjugated second anti-IgG antibody new EBV-specific bands were clearly detectable in all constructs. (Fig. 4)

All expressed proteins display almost the expected size, but the yield varied over a wide range. The proteins encoded by pUC635 and pMF924, seem to be more stably expressable than the non-fusion proteins from pUC924 and pKK378. However, the amount of even the highest expressed protein from pUC635 is too low for a large-scale production since in the Coomassie-stained gel only a very weak band was visible which may be due to the large size of the eukaryotic protein.

Example 3

Immunological assay of the proteins encoded by pUC635 pUC924, pMF924 and pKK378

The host cells transformed with plasmids pUC635, pUC924, pMF924 and pUK378 were cultivated in L-broth supplemented with 50 µg/ml Ampicillin to a cell density of $D_{600}$=0.8. Then, for the induction of the ß-galactosidase the lactose analogon isopropyl-ß-D-thiogalactopyranoside (IPTG; Sigma) was added (final concentration: 1mM). After a further incubation of 1.5 h at 37°C, 1.5 ml of the culture were centrifuged. The bacteria were resuspended in 2oo µl boiling mix (2 % SDS, 5 % mercaptoethanol, 3 % sucrose, 5o mM Tris-HCl, pH 7.o) and heated for 1o min at 1oo°C. 2o µl of the resulting protein extract were separated on a 12.5 % polyacrylamide gel and finally the proteins were visualized by coomassie-blue staining, but since the yield of the expression product is very low, an immunostaining was necessary. Therefore the electrophoretically separated proteins were transferred to a nitrocellulose filter, i.e. a "Western-blot" was prepared (J. Renart, J. Reiser, G.R. Shark "Transfer of proteins from gels to diazobenzyl-methyl-paper and detection with antisera",

Proc. Natl. Acad. Sci. USA 76, p. 3116 (1979), S. Modrow, H. Wolf, "Characterization of herpesvirus saimiri and herpesvirus ateles induced proteins", in: Latent Herpes Infections in Veterinary Medicine, Martinus Nijnoff Publ., p 1o5 (1984)).

The Western-blot was prepared with a current intensity of o.8 A for 3 h in Western-blot buffer (72 g glycin, 15 g Tris, 1 l methanol, $H_2O$ dest. ad 5 l). Then the nitrocellulose was saturated with Cohen buffer for 3 h (o.1 % Ficoll 4oo, 1 % polyvinylpyrrolidone, 1.6 % BSA, o.1 % NP4o, o.o5 % gelatine, o.17 M $H_3BO_3$, 28 mM NaOH, 15o mM NaCl, 6 mM $NaN_3$, pH 8.2) and incubated overnight with 1:5o diluted high titered EBV specific serum from NPC-patients. The serum had been preabsorbed to a bacterial-protein extract (1 ml/$1o^9$ E. coli cells) to reduce the bacterial protein generated background. Afterwards unbound IgG was removed by washing the nitrocellulose filter for 5 h in gelatine buffer (5o mM Tris-HCl, 5 mM EDTA, 15o mM NaCl, o.25 % gelatine, o.5 % Triton, o.2 % SDS, pH 7.5). For visualizing the blotted EBV-specific proteins rabbit anti-human-IgG-antibodies coupled to peroxidase and diluted 1:200 in TN buffer (154 mM NaCl, 1o mM Tris, pH 7.4) was added. After 2 h at RT, unbound rabbit antibodies were removed by washing with gelatine buffer as described above. Finally the peroxidase reaction was carried out in 1oo ml 5o mM Tris-HCl, pH 7.5, by adding 5o mg diaminobenzidine (Sigma) and 4o µl $H_2O_2$ and incubating 1o min. at RT. The results of this experiment are shown in Fig. 5.

Example 4

Purification of the ß-gal : p138 fusion protein encoded by the plasmid pUC635

The clone E.coli K12 JM109 pUC 635 was grown at 37°C in 5oo ml L-broth supplemented with Ampicillin as described above until the $OD_{56o}$ was o.8. The fusion protein synthesis was induced by IPTG (1 mM) and the incubation was continued for another 2 h. Then the cells were collected by centrifugation for 1o min in a GSA rotor (Sorvall) at 5.ooo rpm and they were resuspended in 5o ml 2o mM Tris-HCl, pH 7.5. For lysating the cells, EDTA (5o mM final concentration) and lysozyme (2 mg/ml final concentration) were added and this mixture was incubated for 3o min at 37°C. In the following, the cells were sonicated (Labsonic 151o, Braun) twice for 8 min, Triton X-1oo was added to a final concentration of 3 % and, after further incubation at 37°C for 3o min, insoluble particles of the suspension were pelleted by centrifugation (SS 34 rotor (Sorvall), 2o min, 1o.ooo rpm). The resulting pellet was dissolved in 2o ml of an 8 M urea, 1o mM Tris-HCl, o,5 % ß-mercaptoethanol, pH 7.5, solution and recentrifuged as before.

Finally 80 mg of the proteins were subjected to a column chromatography (Sepharose 2B-Cl (Pharmacia), length 80 cm, diameter 3 cm) with 8 M urea, 10 mM Tris, 0.1 % ß-mercaptoethanol, pH 7.5, buffer. 30 µl of each of the collected 4 ml samples were analyzed in a 15 % PAGE and the fusion protein-containing fractions were pooled.

Example 5

Cloning of p138 subregions coding for antigenic determinants identified by computer analysis

In principle in diagnostic tests only the antigenic determinant subregions of the antigenic protein are needed. Therefore the p138 amino acid sequence was analyzed by a computer programm and the identified subregions of this gene were introduced in suitable vectors. The production of such small proteins has the advantage that these are less vulnerable to rapid changes of antigenicity with decreasing length of the product. Furthermore especially in conjunction with assays for class specific antibodies they will be of diagnostic value.

According to the method of P. Chou and G. Fasman ("Conformational parameters for aminoacids in $\alpha$-helical ß-sheet and random coil regions calculated from proteins", Biochemistry 13, p. 211 (1974)) the calculation of the appropriate secondary structure of a protein caused by its aminoacid sequence (primary structure) is possible. Superimposed on the suggested structure,the program determines the relative hydrophilicity and hydrophobicity. Both data sets are combined and a computer graphic is drawn that shows $\alpha$-helical, ß-sheet, ß-turning and randomly coiled regions of the secondary structure. Thereby the hydrophilic and hydrophobic regions are shown as open and closed circles, respectively.

An example of such computer graphic is shown for the p138 amino acid sequence in Fig. 9.

Based on the assumption that antigenic sites are mainly located in hydrophilic β-turns which are located on the surface of the protein, the region between about amino acid 52o and the carboxy-terminus of p138 should be antigenic. The corresponding DNA sequence is represented by a PstI-fragment of pUC635.

Thus pUC635 was cleaved with PstI and all PstI-fragments were isolated and introduced into PstI-cleaved pUC8, the remaining vector fragment with additional 400 bp (up to the first PstI-site of the p138 coding sequence) was religated (all methods as described in example 2).

The resulting recombinant plasmids were designated pUC P400, pUC P380, pUC P600, pUC P210, pUC P750, and pUC P540, respectively.

The aminoterminal region of the p138 encoding sequence was cloned by digesting the plasmid pBR322-BamA with PstI and HgiAI and inserting said fragment into PstI cleaved pUC9. (J. Messing et al., supra) (methods as described in example 2). The resulting recombinant plasmid is designated pUC HP.

With the exception of pUC HP in which translation stops at the 3' end of the insertion, in all subclones orientation and reading frames relative to pUC8 are correct.

Finally the recombinant plasmids were introduced into E. coli K12 JM 109 cells.

Example 6

Expression of antigenic determinants identified by computer analysis using pUR288 p138 subclones

Since pUC subclones (example 5) somtimes are not stably expressible in bacteria, because they cannot build up a suitable tertiary structure due to their shortness and therefore can be degraded by proteases to a larger extent than complete proteins we constructed recombinant plasmids encoding large fusion proteins using at least a part of the ß-galactosidase encoded by pUR288 (DSM 3415) (U. Rüther et al., supra) by cleaving said PstI-fragment subclones with BamHI and HindIII, isolating the respective fragments and ligating them into BamHI and HindIII cleaved pUR288 (all methods as described in example 2).

The expression was carried out in E.coli K12 JM109. The products were analyzed as described in example 3. After coomassie-blue staining of the gel several large fusion proteins of different size were detected, however, after preparation of a Western-blot, only the products expressed by pUR6oo and pUR54o showed specific reaction with the IgG antibodies mentioned (Fig. 10).

These results are in good agreement with the computer analysis.

Additionally the expression of the clones obtained according to example 5 was carried out according to example 3. The products, too, were analyzed as described in example 3. From the coomassie-blue stained gel it can be taken that only plasmids pUCP600 and pUCP380 code for a stable fusion protein. The Western-blot shows that only pUCP600 derived fusion protein is antigenic (Fig. 11). This fusion protein contains 11 amino acids encoded

by the aminoterminal cloning site, a region encoded by about 600 bp of p138 and carboxyterminal amino acids of the lacZ gene. Thus, the recombinant expression plasmids pUR600 and pUR540 as well as pUCP600 can be used for the production of large and small fusion proteins, respectively, containing an antigenic determinant of EBV-protein p138.

## Example 7

### Application of the protein encoded by plasmid pUCP600 for the stabilization of per se unstable parts of eukaryotic proteins

By means of the experiments of example 6 it was shown that the p138-derived protein parts (regions) are unstable with the exception of the protein encoded by plasmid pUCP600. The second antigenic region from the C-terminus of p138 (P540, see Fig. 9) is not stably expressible using the recombinant pUC-vector pUCP540.
The ability of the P600-region of p138 to stabilize such a per se unstable expression product is shown in this example.

For this purpose it was necessary to remove the 5'-PstI-site of pUCP600 by digesting the plasmid with SstI and HindIII(the SstI site is located about 20 bp 3' from the first PstI site). The p138-related SstI-HindIII fragment was inserted into SstI/HindIII cleaved pUC12 (DSM3422) J. Messing, "New M13 vectors for cloning", in Methods of Enzymology Vol. 101, Part C., R. Wu, L. Großmann and K. Moldoave (eds.), Acad. Press, New York, 1983, 20-78).

Then the resulting recombinant plasmid was digested with EcoRI and PstI. The obtained 600 bp fragment was inserted into plasmid pUC8. The 5'-PstI site was now replaced by an SstI site and thus the reading frame is reconstituted at the 3'- and the 5'-end of the insert (Fig. 12a). The resulting recombinant plasmid pUC601 still expresses a stable product (Fig. 13).

Between the PstI and HindIII site at the 3'-terminus of the EBV-encoded sequence a synthetic oligonucleotide obtained according to known methods coding in frame for 5 arginine and 2 stop codons was inserted as shown in Fig. 12b). The resulting plasmid pUCARG601 encodes the P600 region of p138 fused at its C-terminus to 5 arginine residues.

In a last step the PstI fragment encoding the P540 region of p138 was ligated to the PstI fragment encoding the P600 region of p138 after digestion with PstI. The resulting recombinant plasmid pUCARG1140 encodes a stable protein of about 43kd which contains two antigenic sites of p138 fused in frame. In this fusion protein the protein region P600 stabilizes the protein region P540 (Fig. 13). The arginine residues at the carboxyterminus of the expression product may be used for the purification of the resulting fusion protein as described by Sassenfeld and Brewer (supra) (Fig. 16).

## Example 8

### Construction of the recombinant plasmid pUCARG680

From the plasmid pUCARG1140 a modified version was constructed which lacks 435bp of the p138 encoding region, the C-terminal part of the p600 fragment and the N-terminal part of the p540 fragment. The main antigenic

sites predicted by the computer program are still present. The plasmid was designated as pUCARG680 and its construction was achieved by digesting pUCARG1140 with NcoI (cleavage site coresp onds to bp1841 and bp3243 in Fig. 3). Since the reading frames in the p600 NcoI site and the p540 NcoI site do not fit, the sticky ends were removed with S1-Nuclease.
30 µg of pUCARG1140 were digested with NcoI, the 3.3kb vector-p138 fragment was separated by gelelecrophoresis and purified. 5µg of this DNA fragment were digested with 100 units S1-Nuclease for 15 min at roomtemperature in 100 µl containing 33mM Na-acetate, 50 mM NaCl, 0.03mM $ZnSO_4$, pH 4.5. The digest was stopped by phenol extraction. After precipitation with ethanol the DNA was religated with T4-DNA ligase and used to transform competent E.coli K12 JM109 cells. The resulting clones were screended for the appearance of a new protein with 30kb in size (pUCARG680). The shortened p600/p540 fusion protein encoded by pUCARG680 still reacts as an antigen.

The newly constructed recombinant plasmid pUCARG680 was deposited with the DSM under the deposition number DSM3408.

## Example 9

### Assay of the antigenicity of the fusion protein encoded by plasmid pUCARG1140

Immunoblots with the fusion proteins encoded by the recombinant plasmids pUCARG1140, pUR540, and pUR600 (examples 6 and 7) using individual NPC-sera reveal that the immunological reactions differ in various patients (Fig. 14). In this context it has to be understood that said plasmids encode fusion proteins containing the p138 regions P540 + P600, P540, and P600, respectively (see Fig. 9).

Whereas in NPC serum no.352 the main fraction of the IgG and IgA antibodies is directed to the P540 region, the main fraction in NPC serum no.354 is directed to the P600 region of p138. A representative pool prepared from many sera from NPC patients did not detect additional antigenic sites. The conclusion from this finding is that the antigenic determinants P540 and P600 as encoded by the recombinant plasmids of the present invention are necessary and sufficient to achieve the desired specificity for ELISA tests useful for diagnostic purposes.

## Example 10

### Application of plasmid pUCARG1140 encoded fusion protein for the detection of NPC in ELISA tests

The purified fusion protein encoded by pUCARG1140 was coated on micro-titer plates. Ten individual NPC-sera were tested for their IgG and especially for their IgA reactivity. The IgA-anti-EA titer of these sera was previously determined in conventional immunofluorescence tests. The highest titer found was 1:80. In the ELISA test shown in Fig. 15, two EBV-negative, one NPC-serum pool and ten individual NPC-sera were tested up to a dilution 1:10640. The test was performed according to the usual ELISA protocol. Bound antibodies were detected with peroxidase conjugated mouse anti human IgG, i.e. IgA and peroxidase reaction. All NPC sera show a reaction with the coated antigen (up to 1:2560 in IgA) and no background reaction could be observed in the negative controls. This result indicates that the pUCARG1140 encoded expression product is suitable for the diagnosis and early detection of NPC.

Example 1T

Cloning of a subregion of the gene coding for gp 350 in the vector pUC8

The coding region of gp 250 and gp 350 was mapped to the Bam L-fragment (J. Skare et al., supra) of the EBV B95-8 genome. As both polypeptides share identical regions it was supposed that both proteins are encoded by overlapping reading frames (M. Hummel, D. Thorley-Lawson, E. Kieff, "Epstein-Barr virus DNA fragment encodes messages for the two major envelope glycoproteins (gp 350/300 and gp 220/200)", J. of Virol. 49, p. 413 (1984)). The sequence data of Baer et al. (supra) revealed a large open reading frame including a donor splice site and an acceptor splice site in said Bam L-fragment of the virus genome (Fig. 17 and 18).

It is assumed that gp 350 is the translation product of the unspliced mRNA transcribed from this region and gp 250 is a product of the corresponding spliced mRNA (Fig. 17). Since both products are found in the viral capsids it is assumed that a differential splicing of said mRNA in a manner comparable with the immunoglobulin heavy chain genes (T. Honjo, "Immunoglobulin genes", Ann. Rev. of Immunol. 1, p. 499 (1983)) takes place. During this splicing 630 bp of the mRNA coding for gp 350 are removed to yield the gp 250 coding mRNA (Fig. 17 and 27 (dotted lines)) (R. Baer et al., supra).

Therefore the whole or a part of the reading frame of gp 350 was cloned for finally isolating and producing a gp 350 related product. It should be kept in mind, that not only gp 250 but also gp 350 are highly glycosylated proteins. In contrast, the proteins produced by expression of the recombinant DNA molecules according to the present

invention differ from the respective viral proteins normally occuring in nature. If expression is carried out in prokaryotes unmodified proteins are obtained whereas expression in eukaryotes gives proteins with different patterns of glycosylation or else modifications as compared to the natural product.

The Bam L-fragment was introduced in pBR322, and E. coli K 12 HB 101 was transformed with the recombinant plasmid obtained. (J. Skare, et al., supra)
Instead of the host E.coli K12 HB101 the host bacteria used in the present invention can also be used.

The contents of the publications of M. Hummel et al. (supra), J. R. North et al. (J. R. North, A. J. Morgan, J. L. Thompson, M. A. Epstein, "Purified Epstein-Barr virus $M_r$ 340.000 glycoprotein induces potent virus-neutralizing antibodies when incorporated in liposomes", Proc. Natl. Acad. Sci. USA 79, p. 7504 (1982)) and D. A. Thorley-Lawson and C. A. Poodry ("Identification and Isolation of the Main Component (gp350-gp220) of Epstein-Barr Virus Responsible for Generating Neutralizing Antibodies In Vivo", J. Virol. 43, p. 730 (1984) do not permit predictions that subregions of the gp 250/350 encoding sequence are coding for sufficiently antigenic and/or immunogenic proteins and that these products after selective introduction of these subregions can be stably expressed in prokaryotic and eukaryotic cells. It is therefore surprising that completely unmodified or in a different way modified gp 250/350 related proteins of the present invention are sufficiently active.antigens and/or immunogens. In particular in previous publications it was not excluded that minor carbohydrate residues of the protein contribute significantly to the antigenic or immunogenic potential of this protein.

As shown in Fig.17, a 1.9 **kb** PstI-PstI-fragment of the Bam L-fragment (Hummel et al., supra) contains the part of the gp 350 coding region **beginning at** aminoacid position 232 and ending at aminoacid position 825.

A large scale preparation of the pBR 322-BamL plasmid DNA was done according to the method published by H.C. Birnboim and J. Doly ("A rapid alkaline extraction procedure for screening recombinant plasmid DNA", Nucl. Acids Res. 7, p. 1513 (1979)). 50µg of this DNA were digested for 2 hours at 37°C with 100 units PstI (Boehringer) in 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl, pH 7.5. The digestion was stopped by addition of 1/5 vol. 50 mM EDTA, 60 % sucrose, 2 % bromphenolblue. The resulting solution was electrophoretically separated on a 1 % agarose gel (Seakem, FMC) in Tris-acetate buffer (0.04 M Tris-acetate, 2 mM EDTA, pH 7.6). As a size marker HindIII digested λ-phage DNA (Boehringer) was used. After the electrophoresis at 40 V for 14 hours at room temperature (RT), the gel was stained in Tris-acetate buffer containing 0.5 µg/ml ethidium bromide.

The DNA bands in the gel were visualized by UV-illumination and the 1.9 kb PstI-PstI fragment was isolated as described in example 2.

PstI digested DNA of the vector pUC8 was prepared as described before, except that for inhibition of religation of the vector during the following ligase reaction, the DNA was treated with alkaline phosphatase (0.5 units (Boehringer), 30 min at 37°C).

The concentration of the purified fragments was estimated by electrophoresing 1 µl each in parallel with 100 ng and 500 ng of pUC8-DNA (under conditions described above).

400 ng of the 1.9 kb PstI-PstI-fragment and 100 ng of the PstI digested vector DNA were ligated, E.coli K12 JM109 was transformed with the ligated plasmid DNA and positive clones were identified as described in example 2.

The obtained clone was designated E. coli K12 JM109 pUCLP1.9 and the resulting recombinant plasmid pUCLP1.9, respectively.

Example 12

Cloning of a subregion of the gene coding for gp 350 in the vector pUR290

For the expression of a stable product of the gp 350 subregion said 1.9 kb PstI-PstI-fragment was reclon in the vector pUR290 (DSM 3417) (Fig. 20) (U.Rüther et al., infra). The resulting recombinant plasmid is coding for a fusion protein of an aminoterminal region of the ß-galactosidase, followed by the aminoacids 232 to 825 of gp 350 and aminoacids coded by the cloning-site of pUR290 and pBR322 nucleotide residues. The respective aminoacid sequence is given in figure 21.

50 µg DNA of the plasmid pUCLP 1.9 were digested with 100 units BamHI and HindIII and separated on a 1 % agarose gel as described above. The resulting 1.9 kb BamHI/HindIII fragment that contains only a few more nucleotides than the PstI-PstI-fragment originally introduced into pUC8 was separated from the other resulting fragments on a 1 % agarose gel (as described above). Finally it was isolated from the gel as described above and ligated into BamHI/HindIII digested DNA of the vector pUR290 (U. Rüther, B. Müller-Hill "Easy identification of cDNA clones", EMBO Journal 1o, p. 1791 (1983)) according to the methods described above.

The next step was the transformation of the ß-galactosidase repressor-protein overproducer strain E. coli K12 JM109 with these recombinant DNA molecules. The transformants were plated and analysed as described above, except that the aliquots of the DNA preparations were digested with BamHI/HindIII and EcoRI. The resulting clone, E. coli K12 JM109 pURLP1.9 carries the plasmid pURLP1.9, that is a recombinant of said BamHI-HindIII 1.9 kb fragment of the plasmid pUCLP1.9 and the vector pUR290 (see Fig. 20).

## Example 13

### gp 350 related polypeptides synthesized by E. coli K12 JM109 pURLP1.9

In an overnight culture E. coli K12 JM109 pURLP1.9 was grown at 37°C in 5ml L-broth supplemented with 50 µg/ml Ampicillin. The culture was then diluted to an optical density at 560 nm($OD_{560}$) of 0.4, and 4 ml of this bacteria suspension were incubated at 37°C until an $OD_{560}$ of 0.8. The expression of the genetic information carried by plasmid pURLP1.9 was then induced as described in example 3 and finally the proteins were visualized by coomassie-blue staining as described in example 3.

In comparison with the control experiment, several new proteins, encoded by the plasmid pURLP1.9 and ranging in size from 116 kD to 200 kD, were detected (Fig. 22). The different size of the expression products may be due to incomplete mRNA synthesis or translation. To prove that the new proteins are EBV-related products, all the electrophoretically separated proteins were transferred to a nitrocellulose filter, i.e. a "Western-blot" was prepared according to the method described in example 3. The results of this experiment are shown in Fig. 22.

## Example 14

**Purification of the ß-gal : gp 350 fusion protein encoded by the plasmid pURLP1.9**

The replacement of the natural carboxyterminal amino acid sequence of the ß-galactosidase by a gp 350 related amino acid sequence prevents the formation of ß-galactosidase tetramers. Furthermore the newly expressed fusion protein is present in a high concentration in the bacterial cell. Therefore the fusion protein precipitates in the cytoplasm of the host cell.

According to the method described in example 4 the clone E. coli K12 JM109 pURLP1.9 was used for the production of the corresponding fusion protein.

The results of the several stages of this purification procedure are shown in Fig. 23.

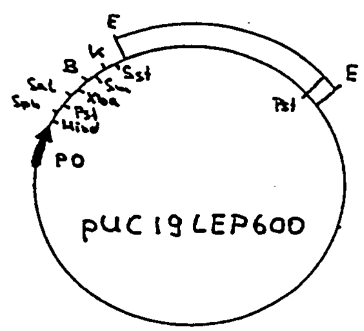
## Example 15

**Expression of selected antigenic epitopes of gp250/350 as ß-galactosidase fusionproteins**

Fig. 24 shows the computer-predicted secondary structure of gp350 together with the relative values of hydrophilic (dark circles) and hydrophobic (grey circles) areas. ß-turns or loop structures are indicated as line turns of 180° ($\alpha$-helices, ß-sheet and coil structures are barely discernable in the scale used). Based on the assumption that antigenic sites are mainly located in ß-turns in an hydrophilic environment, which may be exposes to the surface of the protein, the regions at about aminoacid 50 and aminoacid 740 and 800-830, respectively, are expected to represent antigenic epitopes.

Subcloning and expression of the N-terminus of gp250/350

The EBV BamHI-L fragment which was cloned in pBR322 (see J. Skare et al., supra) was digested with EcoRI (restriction sites at positions 650 and 1284 in the sequence given in Fig. 17), the resulting 634 bp fragment was eluted from an agarose gel after electrophoresis and ligated to EcoRI linearised pUC19 (DSM3425) (Yanisch-Perron et al., Gene 33, 103-119 (1985)). Then, E.coli K12 JM109 was transformed with the ligation products (all steps were carried out as described in Example 2). According to example 2 the recombinant plasmids obtained were tested for the orientation of their insert using suitable restriction enzymes. A recombinant plasmid carrying the insert in the opposite orientation of the reading frame relative to the reading frame of the lacZ gene of the pUC19 plasmid was designated as pUC19LEP600 and used for further cloning:



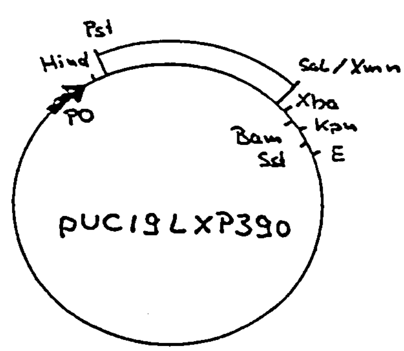

pUC19LEP600 was digested with BamHI and PstI (the BamHI site is derived from pUC19, the PstI site corresponds to position 1248 in Fig. 17), the resulting 600bp fragment was inserted into pUR291 (DSM3418 ) (Rüther, supra), previously digested with BamHI and PstI. The resulting recombinant plasmid pURLEP600 displayed the following sequence in its linker region at the C-terminus of the ß-galactosidase:

pUR291 / pUC19 / gp250/350
ß-gal-TGT CGG GGA TCC CCG GTA CCG GAG CTC GAA TTC CCA TTT---------- ACC
/ pUR291
TGC AGC CAA GCT TAT CGA TGA

The expression of the fusion protein from this recombinant plasmid after IPTG-induction was carried out as described in example 3. The result of this experiment is shown in Fig. 25. From Fig. 25 (lower part) it can be taken that the expression product obtained is recognized as a moderately antigenic protein by a pool of NPC-sera.

Subcloning and expression of the C-terminus of gp250/350

The region covering the antigenic epitopes near the C-terminus which, according to the computer-directed analysis, also is expected to be antigenic, was isolated by digesting the plasmid pUCLP1.9 (see example 11) with XmnI (restriction site at position 2760 in Fig. 17) and HindIII (restriction site in the region derived from the pUC-plasmid). The purified 386 bp fragment was inserted into pUC19 previously digested with HincII and HindIII. The resulting plasmid which was introduced into E.coli K12 JM109 is pUC19LXP390:



purified according to the method given in example 4. An antibody reaction to all three fusion proteins indicates a normal immune status. If there is nor or weaker reaction with the proteins encoded by pURLEP600 and pURLXP390, but reactivity against pURLP1.9 (which contains the intron sequences, see Fig. 27) a chronic EBV-infection is very likely.

IgA antibodies to the membrane protein gp250/350 and to subfragments thereof are absent in the normal population, but present in 58 % of Nasopharyngeal Carcinoma patients when measured in a relatively insensitive immunofluorescence assay. These results are similar to the detection rate of IgA antibodies to EBV specific early antigens in comparable testsystems. In analogy the more sensitive ELISA test brings the detection rate close to 100 % with only minimal increase of false positive results. Therefore the antigens encoded by the newly constructed recombinant plasmids pURLEP600, pURLXP390, and pURLP1.9, respectively, are valuable substances for the initial diagnosis and the control of a therapy of Nasopharyngeal Carcinoma.

## Example 17

### Expression of the N-terminal gp250/350 fragment in the plasmid pUC8

The recombinant plasmid covering the N-terminal region of gp250/350, pUC19LEP600 (see example 15), was digested with BamHI and PstI. The EBV derived fragment was isolated and ligated into pUC8, previously digested with the same enzymes. The sequence in the linker region of the resulting clone, pUCLEP600, is the following:

The insert of pUC19LXP390 was cut out with BamHI and HindIII and ligated into pUR288 digested with the same enzymes. The resulting recombinant plasmid was introduced into E.coli K12 JM109 and was designated as pURLXP390. The sequence in its linker region is as follows:

```
        pUR288    /     pUC19        /        gp350              /   pUC8  /
ß-gal-TGT  CGG GGA TCC TCT AGA GTC AGT TCC CAC-------GTA CTG CAG CCA AGC
pUR288
TTA TCG
```

After IPTG-induction a ß-galactosidase fusion protein was synthesized by said transformed host. In a Western blot the expression product shows a high reactivity with the NPC sera pool (see Fig. 25, lower part).

Example 16

Use of the ß-gal::gp250/350 fusion proteins encoded by the newly constructed recombinant plasmids in diagnostic tests

Jilg et al. (W.Jilg and H. Wolf, "Diagnostic Significance of Antibodies to the Epstein-Barr Virus-Specific Membrane Antigen gp250", The Journal of Infectious Diseases, 152, 222-225(1985)) have shown the validity of gp250 and gp350 as antigens for the determination of the immune status to EBV and especially for the diagnosis of chronic EBV-infections. Persons showing a normal immune response after an EBV-infection possess antibodies against gp250 and gp350, whereas patients suffering from chronic EBV-infection show an immune response only to gp350 which still contains the additional intron sequence (see Fig. 27). The serological status of these persons can be checked in ELISA tests using the three fusion proteins,

pUC8 / pUC19 / gp350

/

ATT ACG AAT TCC CGG GGA TCC CCG GGT ACC GAG CTC GAA TTC CCA

pUC8

TTT-------ACC TGC AGC CAA GCT TAT

After induction with IPTG, the fusion protein encoded by pUCLEP600 is quite stable in the bacterial cells and is recognized as an antigen by the NPC sera pool (see Fig. 26). The bacterial fusion part consists of 14 amino-acids at the N-terminus and 9 at the C-terminus. The value of this protein is its applicability in a vaccine, especially when it is fused with the per se instable second antigenic region from the C-terminus as it was determined with the ß-gal fusion proteins (see example 15).

The inserts of the recombinant expression plasmids and cloning plasmids constructed according to examples 11 and 15 to 17 are summarized in Fig. 27.

## Example 18

### Expression of the N-terminal part of gp250/350 as a p138::gp250/350 fusion protein

The plasmid pUC19LEP600 (see example 15) was digested with PstI and the resulting 600bp fragment was ligated to the PstI linearised plasmid pUCARG601 (see example 7). The gp350-insert was checked to be in the same orientation as the pUCARG601-reading frame and the resulting recombinant plasmid was designated as pUCARG1230. The sequence in the linker region and at the junction sites of the obtained plasmids is the following:

pUC8 / pUC12 / p138 / pUC19

ATG ACC ATG ATT ACG AAT TCG AGC TCT CTG ACC-------ATC CTG CAG GTC GAC TCT AGA

/ gp350 / pUCARG601

GGA TCC CCG GGT ACC GAG CTC GAA TTC CCA TTT-------ACC TGC AGC GTC GTC GTC GTC GTT GAT AAC GTT

After induction with IPTG, E.coli K12 JM109 carrying pUCARG1230 expresses a stable and antigenic protein which consists of antigenic regions from two different proteins, namely p138 and gp250/350 (see Fig. 26).

Furthermore it can be used as antigen in ELISA tests and also for vaccination.

## Example 19

### Neutralisation test with sera derived from rabbits immunized with gp250/350 antigens

Supernatants from B95-8 cells were used to immortalize human umbilical cord blood cells (Lymphocyte fraction from Ficol/Hypaque gradient). 0,5 x $10^6$ lymphocytes were seeded per 0,5 ml microtiter plate well and 50 µl of a cell-free supernatant of B95-8 cells were added and allowed to adsorb for 2 hours at 37°C. After incubation the virus-containing medium was removed, cells were washed with RPMI1640 medium containing 10 % fetal calf serum and incubated in 200 µl of the same medium at 37°C

in a 5 % $CO_2$ atmosphere. Developing colonies of lymphoblastoid cells were evaluated not sooner than three weeks after the start of the experiment and counted as positive transformation.

The neutralizing properties of sera were tested by preincubating for 1 hour under slight agitation aliquotes of the Epstein-Barr Virus containing B95-8 cell supernatant with 20 µl of test serum including the respective preimmunization serum as control in a replicate test before the supernatant was allowed to adsorb to the umbilical cord blood lymphocytes. After removing the inoculum from the cells after 2 hours the maintenance medium (RPMI1640 supplemented with 10 % FCS) was supplemented with 5 % of the respective sera under test for neutralizing activity. The following results were obtained:

| PBS (control) | Virus | Virus | Virus | Virus | Virus |
|---|---|---|---|---|---|
| (no virus) | + | + | + | + | + |
| | EBV negative human serum | EBV positive human serum pool | rabbit preserum | rabbit immune serum 1 | rabbit immune serum 2 |
| no colonies | colonies | no colonies | colonies | no colonies | no colonies |

Example 20

Cloning and expression of antigenic fragments of the virus-capsid protein p150

The coding sequence of the diagnostically relevant protein p 150 (Virus capsid antigen VCA (see Example 1, Fig. 28D) was examined for antigenic sites and subcloned for the expression as ß-galactosidase fusion proteins. The N-terminal region which is expected to encode an antigenic site was obtained by digestion of the Charon 4A phage EB 69-79 (G.N. Buell, D. Reisman, C. Kintner, G. Crouse, and B. Sugden, "Cloning overlapping DNA fragments from the B95-8 strain of Epstein-Barr virus (ATCC CRL 1612) reveals a site of homology to the internal repetition", Journal of Virology 40, 977-982 (1981)) with BamHI and a resulting 1176bp fragment was cloned into the BamHI site of pUC12. From a resulting plasmid with the insertion in the proper orientation a 580bp fragment was excised with XhoI/SalI. The SalI site derives from the pUC12 linker, the XhoI site is located 33bp upstream from the start of p150. This fragment was inserted into pUC8 digested with SalI (SalI and XhoI share the same sticky end sequence). The resulting clones were screened to have the p150 start codon next to the BamHI site. From a proper clone the p150 encoding region was cut out with BamHI and HindIII and cloned into pUR290 digested with BamHI and HindIII (pUR290CXH580). The expression of the ß-Gal::p150 fusion protein from this clone is shown in Figure 29. Its ability to react very well with a NPC serum pool can be taken from Figure 30.

Further, p150::ß-gal fusion contructs were obtained accordingly. For example the subclones pUR290DBX320, pUR292DBB180, pUR290DTT 700, pURDTT740, pUR290DTP680, pUR288DPP320 which are indicated in Figure 31. From the designation of the subclones, the vector used can be taken,e.g. for the construction of subclone pUR290DBX320 the vector pUR290 was used.

From Figure 30 the restriction enzyme sites used for subcloning can also be taken. All clones with the exception of pURDBB180 were constructed by subcloning the desired fragments into pUC8 or pUC12 (see supra) to obtain BamHI and HindIII sites suitable for the cloning to pUR vectors (see supra). pUR292DBB180 was derived by insertion of the 180bp BglII-BglII fragment (see Fig.31) into pUR292 linearized with BamHI. Figures 29 and 30 show their expression and antigenicity.

The ß-gal::p150 fusion protein encoded by pUR290CXH580 and purified according to example 4 reacts in the ELISA test as an EBV specific antigen indicating its applicability in diagnosis. Stable expression was also obtained with the N-terminal fragment of p150 by inserting the 580bp fragment (used for the construction of pUR290CSH580) into pUC18 (DSM 3424) using the BamHI and HindIII site. The resulting clone pUC18CXH580 expresses a stable and antigenic protein of about 25kD in size.

The following deposited plasmids, host bacteria and cell lines were used for the purpose of the present invention. The deposition was affected according to the Budapest treaty

| m.o. | depository | deposition number |
|---|---|---|
| B 95.8 | ATCC | CRL 1612 |
| E.coli K12 JM83 | ATCC | 35607 |
| E.coli K12 BMH71-18 | DSM | 3413 |
| E.coli K12 JM109 | DSM | 3423 |
| pUC8 | DSM | 3420 |
| pUC9 | DSM | 3421 |
| pUC12 | DSM | 3422 |
| pUC19 | DSM | 3425 |
| pUR288 | DSM | 3415 |
| pUR290 | DSM | 3417 |
| pUR291 | DSM | 3418 |
| pUCARG680 | DSM | 3408 |
| pUC18 | DSM | 3424 |

While we have hereinbefore presented a number of embodiments of this invention, it is apparent that our constructions can be alterd to provide other embodiments which utilize DNA sequences of the EBV genome coding for EBV-related antigens and for producing recombinant DNA molecules. It is obvious to those skilled in the art that other DNA sequences may also be used, which are related to said DNA sequences and which may be derived from other EBV serotypes. The EBV is easily obtainable from known natural sources, e. g. from the saliva of infected patients.

It is obvious that for obtaining biologically comparable results other suitable vector/host systems can be used. The invention ist not limited to host/vector systems presently available.

Claims:

1. A DNA sequence of the EBV-genome, characterized in that it corresponds to at least a part of an EBV-related antigenic protein having an aminoacid sequence as shown in Figures 3, 17, and 28.

2. A DNA sequence according to claim 1, characterized in that it corresponds to at least a part of protein p150, p143, p138, p110, p105, p90, p80, p54 or gp250/350.

3. A DNA sequence according to claims 1 or 2, characterized in that it contains additionally the respective regulatory sequences in the 5' and 3' flanks.

4. A DNA sequence hybridizing to a DNA sequence according to anyone of claims 1 to 3 from whatever source obtained including natural, synthetic or semisynthetic sources, which is related by mutations, including nucleotide substitutions, nucleotide deletions, nucleotide insertions and inversions of nucleotide stretches to a DNA sequence according to claims 1 to 3 and which encodes at least a part of a protein according to claim 1.

5. A DNA sequence according to claim 2, characterized in that it is inserted in the recombinant plasmid pUC6130, pUC635, pUCP400, pUCP380, pUCP600, pUCP210, pUCP750, pUCP540, pUCHP, pUC924, pMF924, pKK378, pUR600, pUR540, pUCARG680 or pUCARG1140.

6. A DNA sequence according to claim 2, characterized in that it is inserted in the recombinant plasmid pUCLP1.9, pURLP1.9, pUC19LEP600, pUC19LXP390, pURLXP390, pUCARG1230,pUCLEP600, pUCLXP390 and pURLEP600.

7. A DNA sequence according to claim 2, characterized in that it is inserted in the recombinant plasmid pUR290CXH580, pUR290DBX320, pUR292DBB180, pUR290DTT700, pURDTT740, pUR290DTP680 or pUR288DPP320.

8 . A DNA sequence characterized in that in contains in reading frame at least two regions of a DNA sequence of anyone of claims 1 to 4 derived from a single EBV genome.

9. A DNA sequence according to claim 8 , characterized in that it contains in reading frame at least two regions of a DNA sequence of anyone of claims 1 to 4 derived from different EBV genomes.

10 . A DNA sequence according to anyone of claims 1 to 9 , characterized in that it contains at its 3' end three to fifteen arginine codons positioned in the correct reading frame followed by at least one stop codon.

11. A DNA sequence according to anyone of claims 1 to 6, characterized in that it contains at its 5' end an oligonucleotide encoding an oligopeptide which serves in the resulting polypeptide as a cleavage site for a sequence specific protease or which is cleavable by acid treatment with an acid such as formic acid.

12. A recombinant DNA molecule for cloning, characterized in that it contains a DNA sequence according to anyone of claims 1 to 11.

13. A recombinant DNA molecule for expression, characterized in that it contains a DNA sequence according to anyone of the claims 1 to 11 that is operatively linked to an expresssion control sequence.

14. A recombinant DNA molecule according to claim 13, characterized in that the expression control sequence is selected from the group of the E. coli λ promoter system, the E. coli lac-system, the E. coli ß-lactamase system, the E. coli trp-system, the E. coli lipoprotein promoter, yeasts and other eukaryotic expression control sequences.

15. Vector carrying a part of the p138 encoding DNA sequence the encoded protein of which stabilizes in a fusion protein a protein encoded by a DNA sequence ligated to its 3'-end and carrying a DNA sequence encoding three to fifteen arginine residues followed by at least one stop codon which after insertion of the second DNA sequence is positioned at the 3'-end of this second sequence in the correct reading frame.

16. Vector according to claim 15 which is pUCARG601.

17. A host, characterized in that it is transformed by at least one recombinant DNA molecule according to anyone of claims 12 to 14.

18. A host according to claim 17 selected from the group consisting of strains of E. coli, other bacteria, yeasts, other fungi, animal and human cells.

19. A protein having EBV-related antigenic determinants suitable for diagnosis and therapy of EBV-related diseases, characterized in that it is encoded by a DNA sequence according to anyone of claims 1 to 11.

20. A polyantigen having at least two EBV-related antigenic determinants suitable for diagnosis and therapy of EBV-related diseases, characterized in that it is encoded by a DNA sequence according to anyone of claims 8 and 9.

21. A fusion protein, characterized in that it contains a protein according to claims 19 or 20.

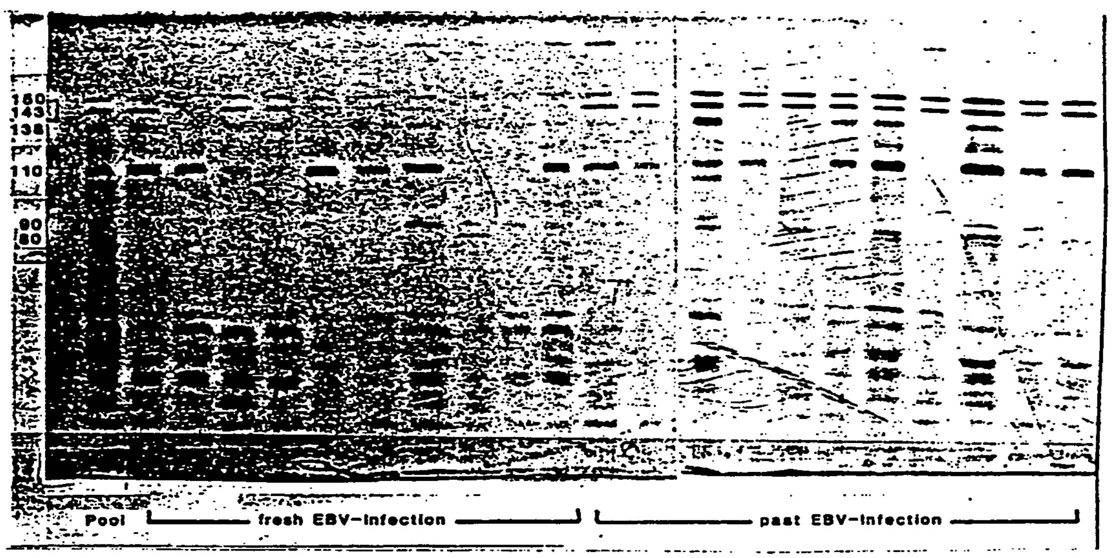
22. A diagnostic composition for the detection of anti-EBV-antibodies, containing at least one protein according to anyone of claims 19 to 21 in an amount sufficient to bind said anti-EBV-antibodies in a sample.

23. A diagnostic composition for the detection of EBV-related diseases, containing at least one DNA sequence according to anyone of claims 1 to 11 in an amount sufficient for hybridization to an EBV-related DNA sequence in a sample.

24. A pharmaceutical composition containing at least one protein according to anyone of claims 19 to 21 in an amount sufficient for stimulating in humans the production of antibodies to EBV and a pharmaceutically acceptable carrier or diluent.

25. A method of preventing EBV infection or therapy of EBV-related diseases comprising administering to a human being the pharmaceutical composition according to claim 24 in an amount sufficient to induce or to modulate an immunoresponse.

Figure 1: Autoradiography of an immunoprecipitation of EBV-specific sera derived from patients suffering from mononucleosis and NPC.



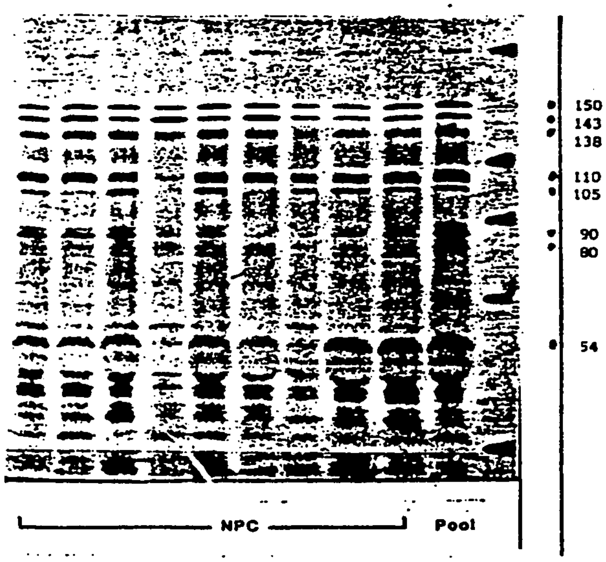
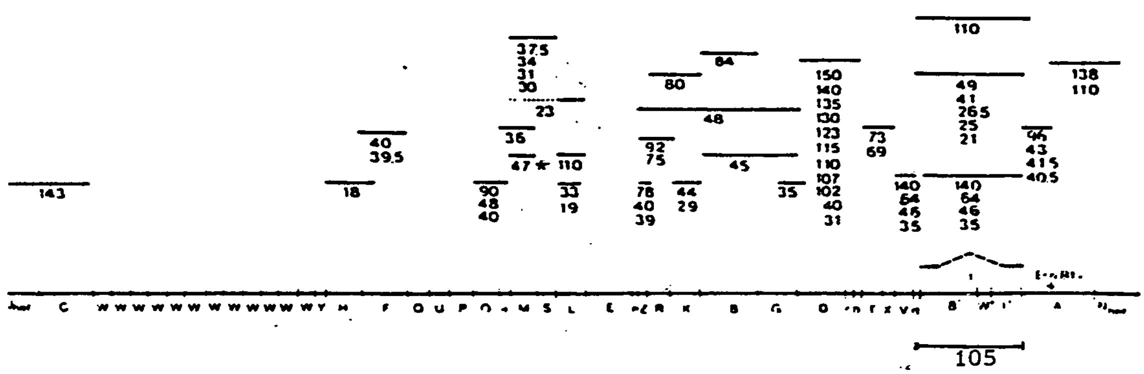



Figure 2

Mapping of mRNA's relative to the EBV B95-8 genome.



47* from translation in vitro correlates with
p54 from in vivo labelling

Figure 3



```
      GTTTGCGAGGCTGGGCGGCATGCCAAGATCGCTGAGACGTCAGTTCCCCGTGACGTGGGC
   1  ---------+---------+---------+---------+---------+---------+   60

      CCTGGCCAGCCTGACTGACTTCCTGAAATCTTTGTAAATGAATAAACAGTGGGTGTTGCG
  61  ---------+---------+---------+---------+---------+---------+  120

      TGATGAGTAAAGTGTAACATTTAATGTGGGACTGGGAGGCCGGGGCGATACCTTGGGCAT
 121  ---------+---------+---------+---------+---------+---------+  180
                HgiAI
      CATGCAGGGTGCACAGACTAGCGAGGATAATCTGGGCAGCCAGAGCCAGCCGGGTCCGTG
 181  ---------+---------+---------+---------+---------+---------+  240
       MetGlnGlyAlaGlnThrSerGluAspAsnLeuGlySerGlnSerGlnProGlyProCy

      CGGCTACATCTACTTTTACCCCCTGGCCACCTACCCTCTTAGGGAGGTGGCCACACTGGG
 241  ---------+---------+---------+---------+---------+---------+  300
      sGlyTyrIleTyrPheTyrProLeuAlaThrTyrProLeuArgGluValAlaThrLeuGl

      GACCGGCTACGCGGGCCACAGGTGCCTGACGGTGCCGCTCCTTTGCGGCATCACCGTGGA
 301  ---------+---------+---------+---------+---------+---------+  360
      yThrGlyTyrAlaGlyHisArgCysLeuThrValProLeuLeuCysGlyIleThrValGl

      GCCGGGCTTCAGCATCAATGTCAAGGCTCTGCACAGGAGGCCCGACCCCAACTGCGGGCT
 361  ---------+---------+---------+---------+---------+---------+  420
      uProGlyPheSerIleAsnValLysAlaLeuHisArgArgProAspProAsnCysGlyLe

      CCTACGCGCTACCTCCTATCACAGGGACATCTACGTGTTCCACAATGCCCATATGGTTCC
 421  ---------+---------+---------+---------+---------+---------+  480
      uLeuArgAlaThrSerTyrHisArgAspIleTyrValPheHisAsnAlaHisMetValPr
                        XhoI
      CCCCATCTTTGAGGGGCCGGGTCTCGAGGCCCTCTGTGGCGAGACCAGGGAGGTGTTTGG
 481  ---------+---------+---------+---------+---------+---------+  540
      oProIlePheGluGlyProGlyLeuGluAlaLeuCysGlyGluThrArgGluValPheGl

      GTACGACGCCTACAGCGCCCTACCGAGGGAAAGCTCCAAGCCGGGGGACTTCTTCCCCGA
 541  ---------+---------+---------+---------+---------+---------+  600
      yTyrAspAlaTyrSerAlaLeuProArgGluSerSerLysProGlyAspPhePheProGl

      AGGGCTAGATCCCTCTGCCTACCTGGGGGCGGTGGCAATAACCGAGGCCTTCAAGGAGCG
 601  ---------+---------+---------+---------+---------+---------+  660
      uGlyLeuAspProSerAlaTyrLeuGlyAlaValAlaIleThrGluAlaPheLysGluAr

      ACTCTACAGCGGAAACCTGGTGGCCATTCCATCGTTAAAACAGGAGGTAGCGGTGGGGCA
 661  ---------+---------+---------+---------+---------+---------+  720
      gLeuTyrSerGlyAsnLeuValAlaIleProSerLeuLysGlnGluValAlaValGlyGl

      GTCTGCGAGCGTTAGGGTCCCGCTCTACGACAAGGAGGTGTTCCCAGAGGGCGTGCCCCA
 721  ---------+---------+---------+---------+---------+---------+  780
      nSerAlaSerValArgValProLeuTyrAspLysGluValPheProGluGlyValProGl

      GCTCCGCCAGTTTTACAACTCGGACCTCAGCCGCTGCATGCACGAGGCGCTGTACACCGG
 781  ---------+---------+---------+---------+---------+---------+  840
      nLeuArgGlnPheTyrAsnSerAspLeuSerArgCysMetHisGluAlaLeuTyrThrGl

      GCTGGCGCAGGCGCTGCGCGTCCGACGGGTGGGCAAGCTGGTGGAGCTGCTGGAGAAGCA
 841  ---------+---------+---------+---------+---------+---------+  900
      yLeuAlaGlnAlaLeuArgValArgArgValGlyLysLeuValGluLeuLeuGluLysGl
          PstI
      GAGCCTGCAGGACCAGGCCAAGGTGGCCAAGGTGGCCCCCCTCAAGGAGTTCCCAGCCTC
 901  ---------+---------+---------+---------+---------+---------+  960
```

```
      nSerLeuGlnAspGlnAlaLysValAlaLysValAlaProLeuLysGluPheProAlaSe

      AACCATCAGTCACCCGGACTCGGGAGCCTTAATGATTGTGGACAGCGCGGCATGCGAGCT
 961  ---------+---------+---------+---------+---------+---------+  1020
      rThrIleSerHisProAspSerGlyAlaLeuMetIleValAspSerAlaAlaCysGluLe

      GGCGGTGAGCTACGCACCCGCCATGCTGGAGGCCTCGCACGAGACCCCGGCCAGCCTCAA
1021  ---------+---------+---------+---------+---------+---------+  1080
      uAlaValSerTyrAlaProAlaMetLeuGluAlaSerHisGluThrProAlaSerLeuAs

      CTACGACTCGTGGCCCCTGTTTGCCGACTGTGAGGGTCCAGAGGCCCGTGTGGCTGCGTT
1081  ---------+---------+---------+---------+---------+---------+  1140
      nTyrAspSerTrpProLeuPheAlaAspCysGluGlyProGluAlaArgValAlaAlaLe
                                                   BglII
      ACACCGATATAATGCCAGCCTGGCCCCCCACGTGTCCACGCAGATCTTTGCCACCAATTC
1141  ---------+---------+---------+---------+---------+---------+  1200
      uHisArgTyrAsnAlaSerLeuAlaProHisValSerThrGlnIlePheAlaThrAsnSe

      CGTCCTCTACGTCTCGGGGGTCTCGAAGTCAACCGGTCAGGGCAAGGAGAGTCTCTTTAA
1201  ---------+---------+---------+---------+---------+---------+  1260
      rValLeuTyrValSerGlyValSerLysSerThrGlyGlnGlyLysGluSerLeuPheAs
                                PstI
      CAGTTTCTACATGACCCACGGCCTGGGGACCCTGCAGGAGGGGACCTGGGACCCCTGCCG
1261  ---------+---------+---------+---------+---------+---------+  1320
      nSerPheTyrMetThrHisGlyLeuGlyThrLeuGlnGluGlyThrTrpAspProCysAr

      CCGACCCTGCTTCTCGGGCTGGGGTGGGCCAGACGTGACCGGAACCAACGGTCCGGGAAA
1321  ---------+---------+---------+---------+---------+---------+  1380
      gArgProCysPheSerGlyTrpGlyGlyProAspValThrGlyThrAsnGlyProGlyAs

      CTACGCTGTGGAGCACCTGGTCTATGCGGCCTCCTTCTCGCCCAACCTTCTTGCCCGCTA
1381  ---------+---------+---------+---------+---------+---------+  1440
      nTyrAlaValGluHisLeuValTyrAlaAlaSerPheSerProAsnLeuLeuAlaArgTy
          PstI                     SstI
      TGCCTACTACCTGCAGTTTTGCCAGGGACAGAAGAGCTCTCTGACCCCGGTGCCGGAGAC
1441  ---------+---------+---------+---------+---------+---------+  1500
      rAlaTyrTyrLeuGlnPheCysGlnGlyGlnLysSerSerLeuThrProValProGluTh

      GGGCAGCTACGTGGCGGGGGCGGCCGCCAGTCCCATGTGCTCGCTCTGCGAGGGCCGGGC
1501  ---------+---------+---------+---------+---------+---------+  1560
      rGlySerTyrValAlaGlyAlaAlaAlaSerProMetCysSerLeuCysGluGlyArgAl

      CCCGGCCGTGTGCCTGAACACGCTCTTCTTTAGGCTGAGGGACCGCTTCCCCCCCGTCAT
1561  ---------+---------+---------+---------+---------+---------+  1620
      aProAlaValCysLeuAsnThrLeuPhePheArgLeuArgAspArgPheProProValMe

      GTCCACGCAGCGGAGGGACCCCTATGTGATCTCGGGGGCCTCGGGCTCCTACAACGAGAC
1621  ---------+---------+---------+---------+---------+---------+  1680
      tSerThrGlnArgArgAspProTyrValIleSerGlyAlaSerGlySerTyrAsnGluTh

      GGACTTTTTGGGCAACTTTCTCAACTTCATCGATAAGGAGGACGACGGGCAGCGGCCGGA
1681  ---------+---------+---------+---------+---------+---------+  1740
      rAspPheLeuGlyAsnPheLeuAsnPheIleAspLysGluAspAspGlyGlnArgProAs

      CGACGAGCCCCGCTACACCTACTGGCAGCTGAACCAGAACCTGCTGGAGCGGCTGTCTCG
1741  ---------+---------+---------+---------+---------+---------+  1800
      pAspGluProArgTyrThrTyrTrpGlnLeuAsnGlnAsnLeuLeuGluArgLeuSerAr

      GCTGGGCATAGACGCTGAAGGAAAGCTAGAGAAGGAGCCCCATGGCCCGCGTGACTTTGT
1801  ---------+---------+---------+---------+---------+---------+  1860
      gLeuGlyIleAspAlaGluGlyLysLeuGluLysGluProHisGlyProArgAspPheVa
```

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```
     CAAGATGTTCAAGGACGTGGATGCGGCGGTGGACGCCGAAGTGGTCCAGTTTATGAACAG
1861 ---------+---------+---------+---------+---------+---------+ 1920
     lLysMetPheLysAspValAspAlaAlaValAspAlaGluValValGlnPheMetAsnSe

     CATGGCCAAGAACAACATCACCTACAAGGACCTGGTCAAGAGCTGCTACCACGTGATGCA
1921 ---------+---------+---------+---------+---------+---------+ 1980
     rMetAlaLysAsnAsnIleThrTyrLysAspLeuValLysSerCysTyrHisValMetGl

     GTACTCGTGCAACCCCTTTGCGCAGCCCGCCTGCCCCATCTTCACCCAGCTGTTTTACCG
1981 ---------+---------+---------+---------+---------+---------+ 2040
     nTyrSerCysAsnProPheAlaGlnProAlaCysProIlePheThrGlnLeuPheTyrAr
                         PstI
     CTCACTGCTGACCATCCTGCAGGACATCTCCCTGCCCATCTGTATGTGCTATGAGAATGA
2041 ---------+---------+---------+---------+---------+---------+ 2100
     gSerLeuLeuThrIleLeuGlnAspIleSerLeuProIleCysMetCysTyrGluAsnAs

     CAACCCCGGGCTTGGCCAGAGCCCCCCAGAGTGGCTAAAGGGTCACTACCAGACGCTGTG
2101 ---------+---------+---------+---------+---------+---------+ 2160
     pAsnProGlyLeuGlyGlnSerProProGluTrpLeuLysGlyHisTyrGlnThrLeuCy

     CACCAACTTTAGGAGCCTGGCCATCGACAAGGGGGTCCTCACGGCCAAGGAGGCCAAGGT
2161 ---------+---------+---------+---------+---------+---------+ 2220
     sThrAsnPheArgSerLeuAlaIleAspLysGlyValLeuThrAlaLysGluAlaLysVa
                                                         Pstl
     GGTGCATGGGGAGCCCACCTGCGACCTGCCAGACCTGGACGCGGCCCTGCAGGGCCGGGT
2221 ---------+---------+---------+---------+---------+---------+ 2280
     lValHisGlyGluProThrCysAspLeuProAspLeuAspAlaAlaLeuGlnGlyArgVa

     GTACGGCCGGCGGCTGCCTGTGCGCATGTCCAAGGTGCTGATGCTGTGCCCCAGGAACAT
2281 ---------+---------+---------+---------+---------+---------+ 2340
     lTyrGlyArgArgLeuProValArgMetSerLysValLeuMetLeuCysProArgAsnIl

     CAAGATCAAGAACAGGGTGGTCTTCACGGGGGAGAATGCCGCCCTCCAGAACAGCTTCAT
2341 ---------+---------+---------+---------+---------+---------+ 2400
     eLysIleLysAsnArgValValPheThrGlyGluAsnAlaAlaLeuGlnAsnSerPheIl

     CAAGTCCACTACCAGGAGGGAGAACTACATCATCAACGGGCCCTACATGAAATTCCTCAA
2401 ---------+---------+---------+---------+---------+---------+ 2460
     eLysSerThrThrArgArgGluAsnTyrIleIleAsnGlyProTyrMetLysPheLeuAs

     CACCTACCACAAGACCCTATTCCCGGACACTAAGCTCTCAAGCCTGTACCTGTGGCACAA
2461 ---------+---------+---------+---------+---------+---------+ 2520
     nThrTyrHisLysThrLeuPheProAspThrLysLeuSerSerLeuTyrLeuTrpHisAs

     CTTTTCCAGGCGGCGCTCGGTCCCTGTCCCCAGCGGGGCCAGCGCGGAGGAGTACTCTGA
2521 ---------+---------+---------+---------+---------+---------+ 2580
     nPheSerArgArgArgSerValProValProSerGlyAlaSerAlaGluGluTyrSerAs

     CCTGGCCCTCTTTGTGGACGGGGGCTCCCGGGCCCACGAAGAGAGCAACGTCATAGATGT
2581 ---------+---------+---------+---------+---------+---------+ 2640
     pLeuAlaLeuPheValAspGlyGlySerArgAlaHisGluGluSerAsnValIleAspVa

     GGTGCCTGGCAACCTGGTCACTTACGCCAAGCAGAGGCTCAACAACGCCATCCTGAAGGC
2641 ---------+---------+---------+---------+---------+---------+ 2700
     lValProGlyAsnLeuValThrTyrAlaLysGlnArgLeuAsnAsnAlaIleLeuLysAl

     GTGCGGCCAGACCCAGTTCTACATCAGCCTGATTCAGGGACTGGTGCCGAGGACGCAGTC
2701 ---------+---------+---------+---------+---------+---------+ 2760
     aCysGlyGlnThrGlnPheTyrIleSerLeuIleGlnGlyLeuValProArgThrGlnSe
```

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```
      GGTGCCCGCCCGTGACTACCCCCACGTACTGGGCACGCGGGCGGTGGAGTCGGCAGCGGC
2761  ---------+---------+---------+---------+---------+---------+  2820
      rValProAlaArgAspTyrProHisValLeuGlyThrArgAlaValGluSerAlaAlaAl

      CTACGCGGAGGCCACCTCCTCCCTTACTGCGACCACGGTGGTCTGCGCGGCCACAGACTG
2821  ---------+---------+---------+---------+---------+---------+  2880
      aTyrAlaGluAlaThrSerSerLeuThrAlaThrThrValValCysAlaAlaThrAspCy

      TCTTAGCCAGGTCTGCAAGGCCCGTCCGGTTGTCACGCTGCCAGTGACCATCAACAAGTA
2881  ---------+---------+---------+---------+---------+---------+  2940
      sLeuSerGlnValCysLysAlaArgProValValThrLeuProValThrIleAsnLysTy

      CACGGGGGTCAACGGCAACAACCAGATATTCCAGGCCGGGAACCTGGGATACTTTATGGG
2941  ---------+---------+---------+---------+---------+---------+  3000
      rThrGlyValAsnGlyAsnAsnGlnIlePheGlnAlaGlyAsnLeuGlyTyrPheMetGl
                               PstI
      CCGGGGCGTGGACAGGAACCTGCTGCAGGCCCCCGGGGCTGGGCTGCGCAAGCAGGCCGG
3001  ---------+---------+---------+---------+---------+---------+  3060
      yArgGlyValAspArgAsnLeuLeuGlnAlaProGlyAlaGlyLeuArgLysGlnAlaGl

      GGGCTCTTCCATGCGGAAGAAGTTTGTCTTTGCCACCCCCACCCTAGGGTTGACCGTGAA
3061  ---------+---------+---------+---------+---------+---------+  3120
      yGlySerSerMetArgLysLysPheValPheAlaThrProThrLeuGlyLeuThrValLy

      GCGCCGGACCCAAGCCGCGACCACATATGAGATTGAGAACATCAGGGCTGGCCTGGAGGC
3121  ---------+---------+---------+---------+---------+---------+  3180
      sArgArgThrGlnAlaAlaThrThrTyrGluIleGluAsnIleArgAlaGlyLeuGluAl

      CATTATATCACAAAAACAGGAGGAAGACTGTGTGTTTGATGTGGTGTGCAACCTTGTGGA
3181  ---------+---------+---------+---------+---------+---------+  3240
      aIleIleSerGlnLysGlnGluGluAspCysValPheAspValValCysAsnLeuValAs

      TGCCATGGGCGAGGCATGCGCCTCGCTGACTAGGGACGACGCGGAGTACTTATTGGGCCG
3241  ---------+---------+---------+---------+---------+---------+  3300
      pAlaMetGlyGluAlaCysAlaSerLeuThrArgAspAspAlaGluTyrLeuLeuGlyAr

      CTTCTCCGTCCTGGCGGACAGCGTCCTAGAAACCCTGGCGACCATTGCCTCCAGCGGGAT
3301  ---------+---------+---------+---------+---------+---------+  3360
      gPheSerValLeuAlaAspSerValLeuGluThrLeuAlaThrIleAlaSerSerGlyIl

      AGAGTGGACGGCGGAGGCCGCTCGGGACTTTCTGGAGGGAGTGTGGGGTGGGCCCGGGGC
3361  ---------+---------+---------+---------+---------+---------+  3420
      eGluTrpThrAlaGluAlaAlaArgAspPheLeuGluGlyValTrpGlyGlyProGlyAl

      AGCCCAGGACAACTTTATCAGCGTGGCCGAGCCGGTCAGCACCGCGTCGCAGGCCTCGGC
3421  ---------+---------+---------+---------+---------+---------+  3480
      aAlaGlnAspAsnPheIleSerValAlaGluProValSerThrAlaSerGlnAlaSerAl

      CGGGCTGCTGCTGGGTGGAGGAGGGCAGGGCTCCGGGGGCAGACGCAAGCGCCGTCTGGC
3481  ---------+---------+---------+---------+---------+---------+  3540
      aGlyLeuLeuLeuGlyGlyGlyGlyGlnGlySerGlyGlyArgArgLysArgArgLeuAl
                XhoI
      CACCGTTCTCCCCGGACTCGAGGTCTAGAGACCCCTGGGGCGGCGATGTCGGGGCTGCTG
3541  ---------+---------+---------+---------+---------+---------+  3600
      aThrValLeuProGlyLeuGluValEnd

      GCGGCGGCGTACAGCCAGGTGTACGCCCTGGCGGTTGAGCTGAGCGTGTGCACCCGGCTG
3601  ---------+---------+---------+---------+---------+---------+  3660

      GACCCCCGGAGTCTGGACGTGGCTGCGGTGGTGCGCAACGCCGGCCTGCTGGCCGAGCTG
3661  ---------+---------+---------+---------+---------+---------+  3720
```

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```
       GAGGCCATCCTCCTTCCCCGTTTGAGACGGCAGAATGACCGTGCATGCAGCGCCCTGTCC
3721   ---------+---------+---------+---------+---------+---------+   3780
                                  XhoI
       CTGGAGCTGGTGCACCTGCTAGAGAACTCGAGAGAGGCCTCTGCCGCGCTGCTCGCCCCT
3781   ---------+---------+---------+---------+---------+---------+   3840

       GGTAGAAAGGG
3841   ---------+--  3851
```

Figure 4:
Restriction map of the plasmids pUC635 and pUC6130.

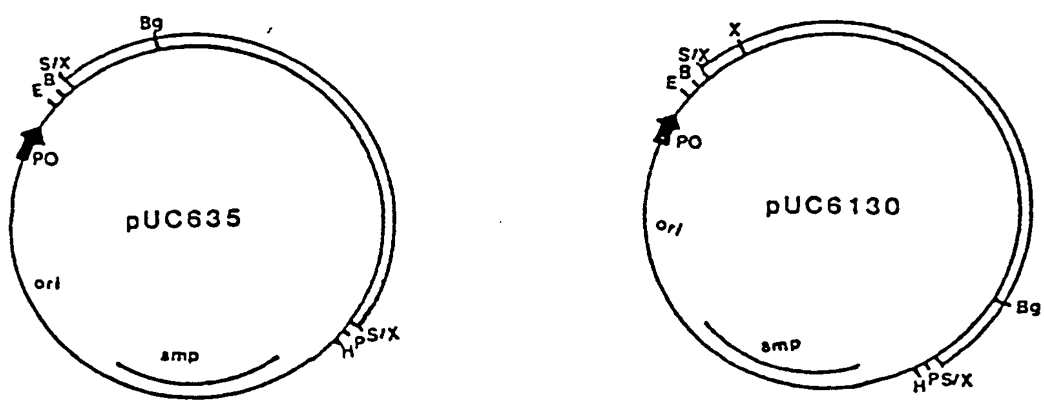


Figure 5:

Expression of the p138 fusion protein encoded by pUC635, pUC924, pMF924, and pKK378

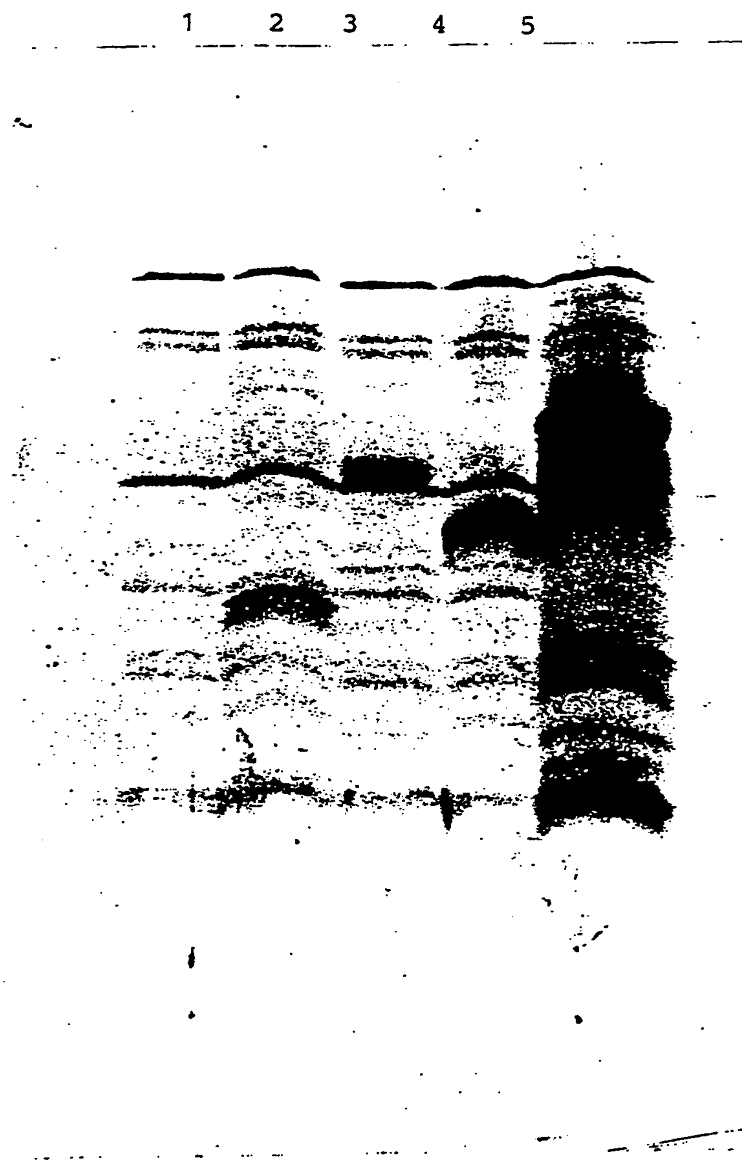


Figure 6:
Restriction map of the plasmid pUC924

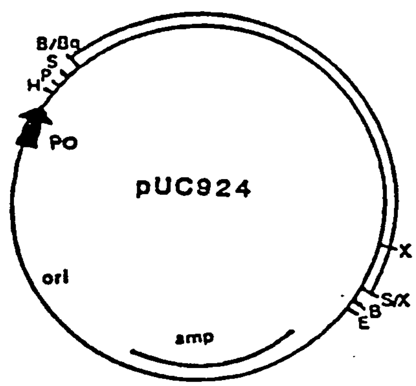


Figure 7:
Restriction map of the plasmid pMF924.

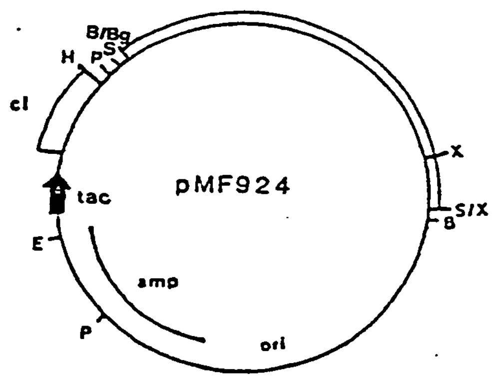


Figure 8:
Restriction map of the plasmid pKK378.

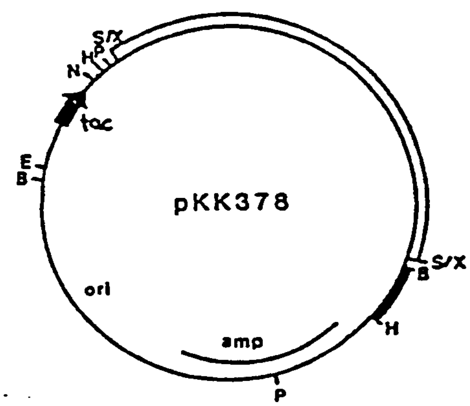


Figure 9:
Secondary structures of p138.

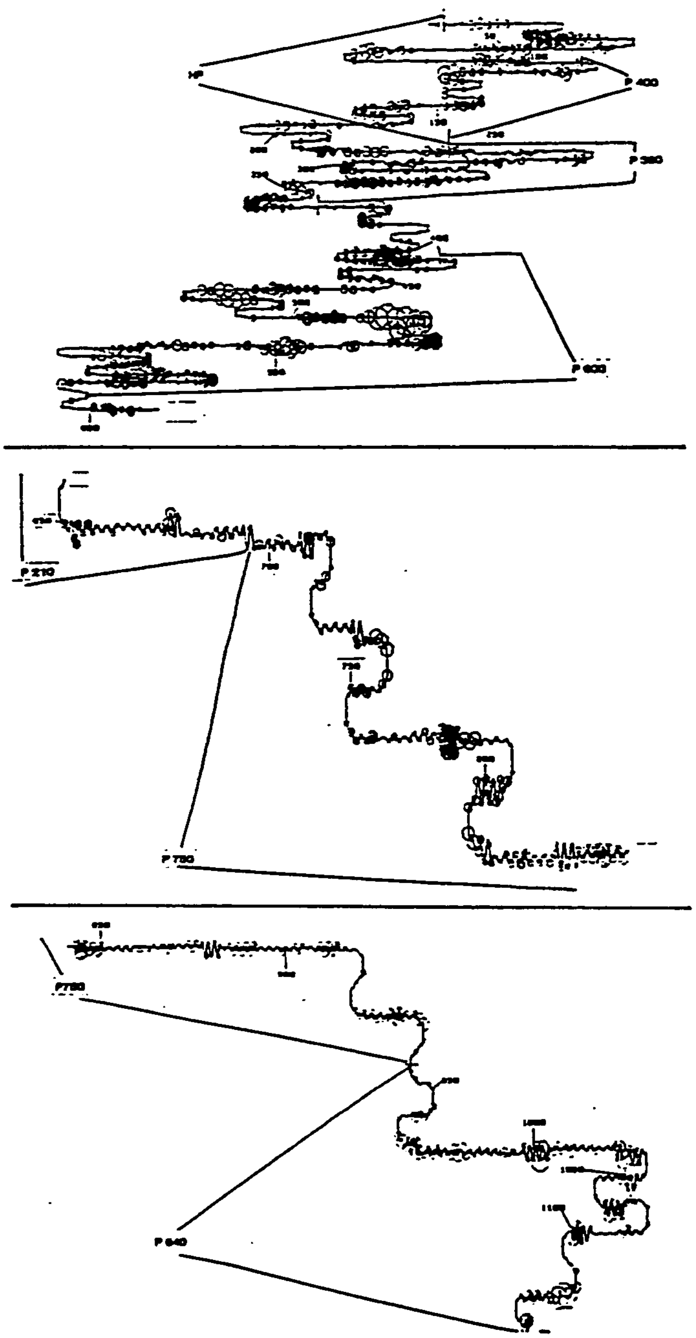
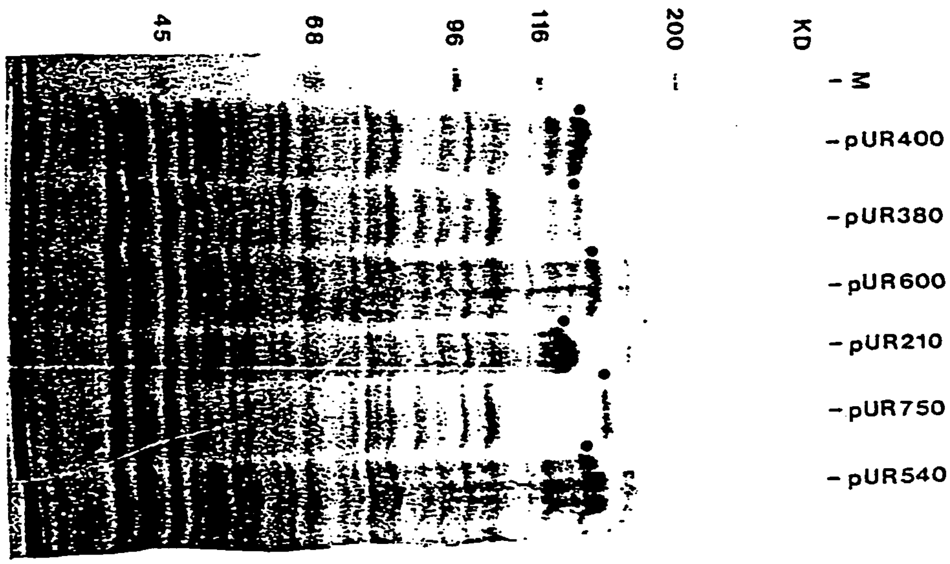


Figure 10:

Expression products of bacteria transformed with the pUR-carrying PstI fragments of p138.

A




B

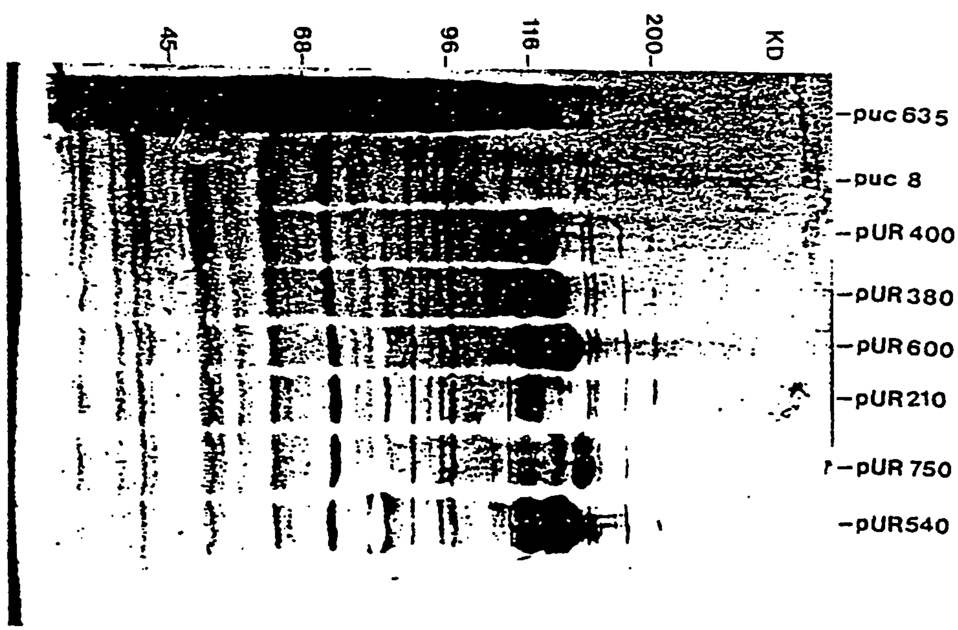


Figure 11:
Expression products of bacteria transformed with the pUC subclones carrying PstI-fragments of p138.

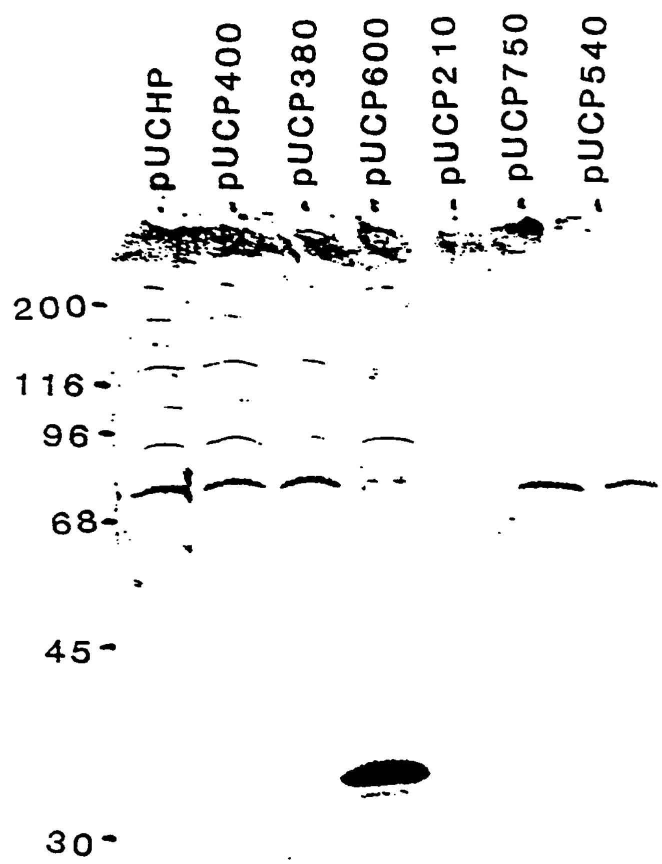
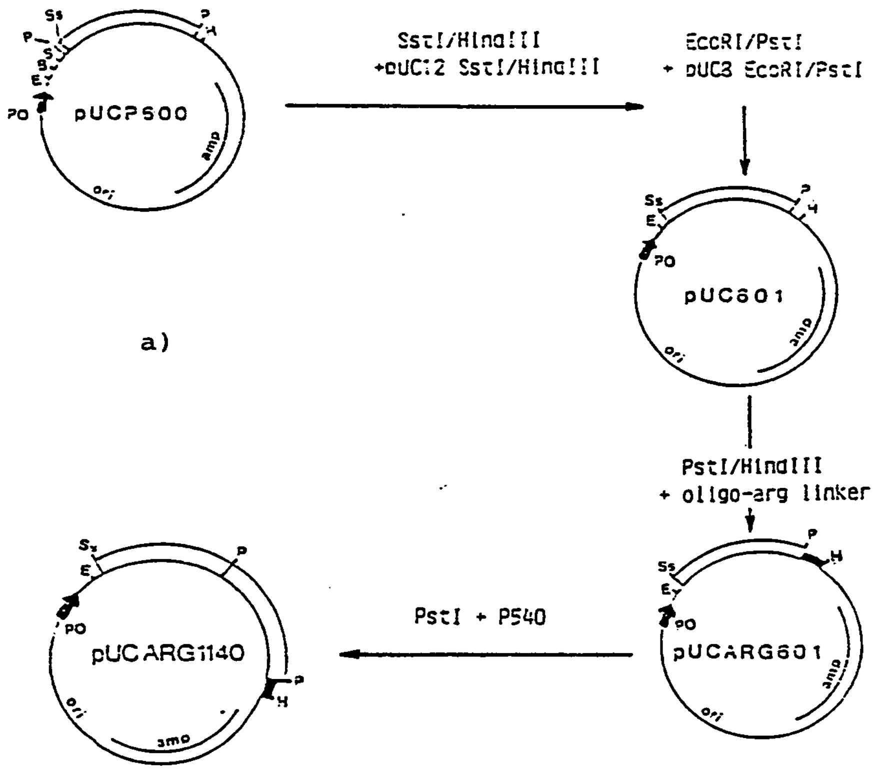


Figure 12:
Construction scheme for pUCARG1140 encoding both antigenic sites found by expression as ß-gal fusion proteins



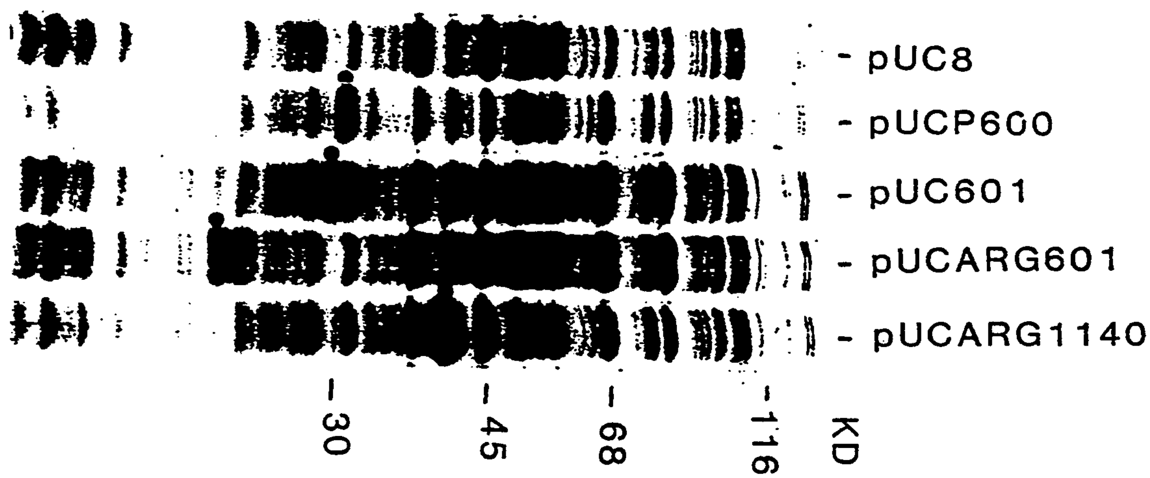

a)

b)

```
PstI                                    HindIII
    5'                                 3'
     G CGT CGT CGT CGT CGT TGA TA
AC GTC GCA GCA GCA GCA GCA ACT ATT CGA
       Arg Arg Arg Arg Arg stop stop
```

Figure 13:

IPTG-induced expression of the plasmids pUC600, pUC601, pUCARG601 and pUCARG1140 with pUC8 as a control



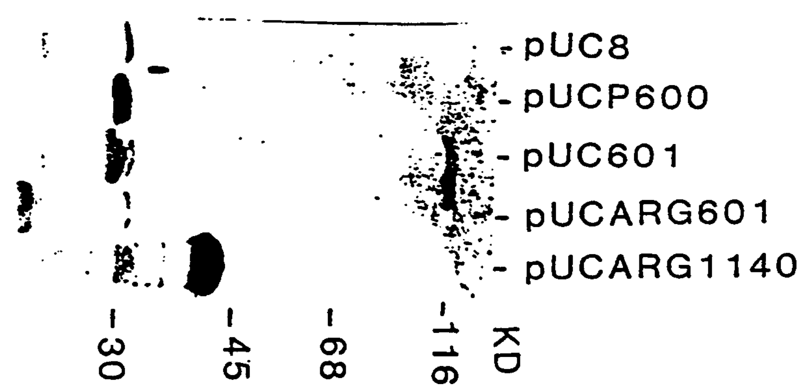
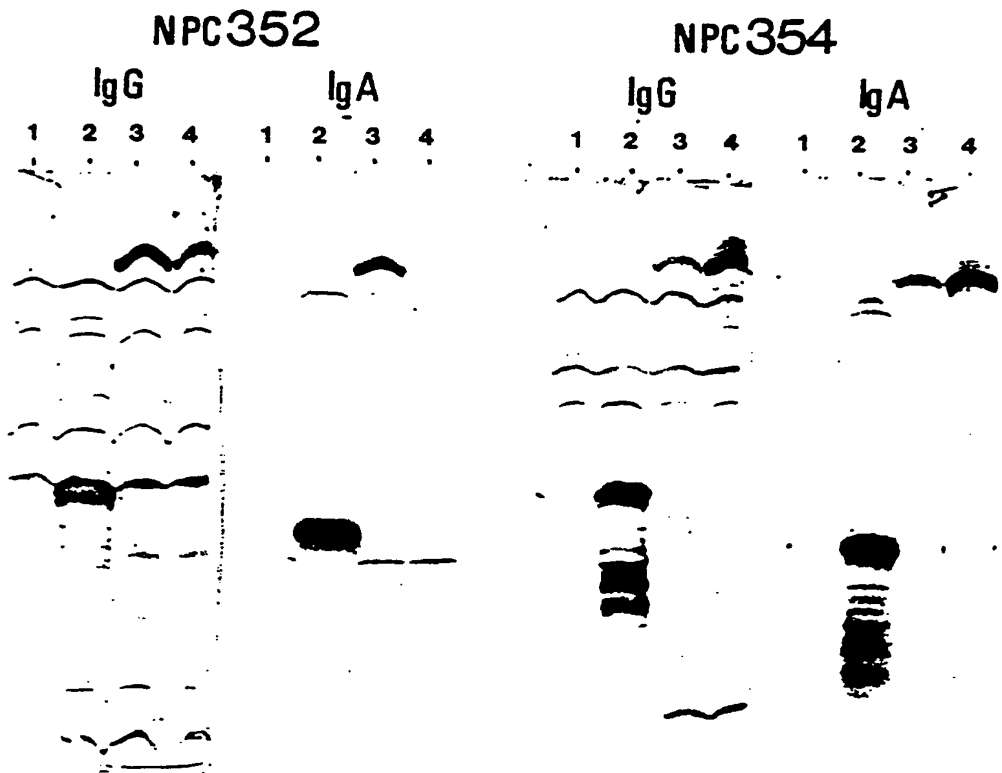



Figure 14:

Distribution and reactivity of the IgG and IgA antibodies of individual NPC-sera against the two epitopes detected in p138




1 -pUR 288
2 -pUCARG 1140
3 -pUR 540
4 -pUR 600

Figure 15:

ELISA test using the protein encoded by pUCARG1140 as antigen

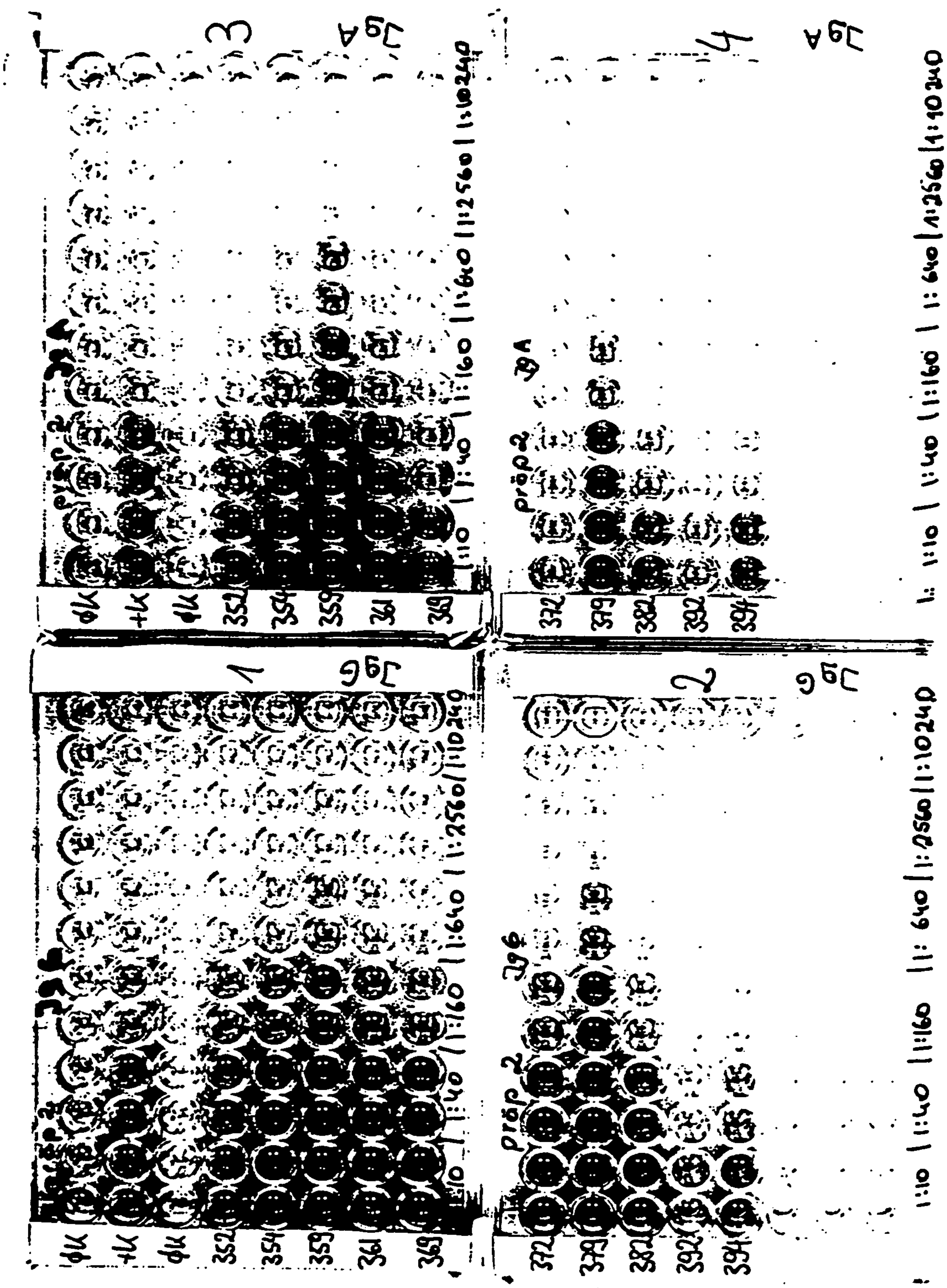


Figure 16:

Purification of proteins carrying oligo-arginine peptides at their carboxy-terminus.

```
  5'G CGT CGT CGT CGT CGT TGA TA 3'
AC GTC GCA GCA GCA GCA GCA ACT ATT CGA          Arg-linker

A         Arg Arg Arg Arg Arg StopStop

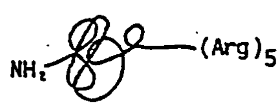
5'GAC CTG CA                       A GCT TGG CAC 3'
  CTG G                                ACC GTG    pUC 8

    Pst I                           Hind III
```

B

Induction with IPTG

Transcription/Translation




Lysis, Zentrifugation

↓

Pellet+ Urea

↓

SP Sephadex C-25-Chromatography
NaCl-Gradient

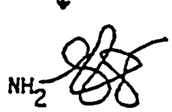
↓

Elution of the expressionproduct in high salt

↓

Digest with Carboxypeptidase B

↓




Arg
Arg
Arg
Arg
Arg

↓

DEAE-Chromatography

↓

Elution of the expressionproduct in low salt

Figure 17

```
       GGATCCGAAAAACTGGTCTATGGCTCGTGTGTCGATGCGCTGAAACCAACGGCAACAAAT
   1   ---------+---------+---------+---------+---------+---------+   60

       TACTTACCTTGTTGTTGTGTGATGGGTAAAAACACACATCACACACTTAGGCCATAGGGA
  61   ---------+---------+---------+---------+---------+---------+   120

       TGCTCACCGTAGCCGCGGCTCCAATCGCTTGAAGAAGTGTTCTTAGATCTAGTGGAAACC
 121   ---------+---------+---------+---------+---------+---------+   180

       TGCGGAGAATGGCTTCTCGCCCAGGGAGATCCGGCTGGGGTGGGAGCATGGGTCGTGCTG
 181   ---------+---------+---------+---------+---------+---------+   240

       GAGCTGACCCACCGGCATCATGATCGACCCGCTTTCTCTTCGTACCCTTCTGGGCCGGCT
 241   ---------+---------+---------+---------+---------+---------+   300

       CCAGGTGGGCATCTTCTGCTTCCTTTTCTGAGCTGCTATCTGATAACTCTATGAGGACAT
 301   ---------+---------+---------+---------+---------+---------+   360

       TTTCCCAATCTCCCGCCGATACCTGTTCCTGCACAACCGAGGTAGATGGGACTTCTTCTT
 361   ---------+---------+---------+---------+---------+---------+   420

       CCATGTTGTCATCCAGGGCCGGGGGACCCGGCCTGTCCTTGTCCATTTTGTCTGCAACAA
 421   ---------+---------+---------+---------+---------+---------+   480

                                           .......
       AAGTGTGACTCACCAACACCGCACCCCCCTTGTACCTATTAAAGAGGATGCTGCCTAGAA
 481   ---------+---------+---------+---------+---------+---------+   540

       ATCGGTGCCGAGACAATGGAGGCAGCCTTGCTTGTGTGTCAGTACACCATCCAGAGCCTG
 541   ---------+---------+---------+---------+---------+---------+   600
                      MetGluAlaAlaLeuLeuValCysGlnTyrThrIleGlnSerLeu
                                                      EcoRI
       ATCCATCTCACGGGTGAAGATCCTGGTTTTTTCAATGTTGAGATTCCGGAATTCCCATTT
 601   ---------+---------+---------+---------+---------+---------+   660
       IleHisLeuThrGlyGluAspProGlyPhePheAsnValGluIleProGluPheProPhe

       TACCCCACATGCAATGTTTGCACGGCAGATGTCAATGTAACTATCAATTTCGATGTCGGG
 661   ---------+---------+---------+---------+---------+---------+   720
       TyrProThrCysAsnValCysThrAlaAspValAsnValThrIleAsnPheAspValGly

       GGCAAAAAGCATCAACTTGATCTTGACTTTGGCCAGCTGACACCCCATACGAAGGCTGTC
 721   ---------+---------+---------+---------+---------+---------+   780
       GlyLysLysHisGlnLeuAspLeuAspPheGlyGlnLeuThrProHisThrLysAlaVal

       TACCAACCTCGAGGTGCATTTGGTGGCTCAGAAAATGCCACCAATCTCTTTCTACTGGAG
 781   ---------+---------+---------+---------+---------+---------+   840
       TyrGlnProArgGlyAlaPheGlyGlySerGluAsnAlaThrAsnLeuPheLeuLeuGlu
                                                 HindIII
       CTCCTTGGTGCAGGAGAATTGGCTCTAACTATGCGGTCTAAGAAGCTTCCAATTAACGTC
 841   ---------+---------+---------+---------+---------+---------+   900
       LeuLeuGlyAlaGlyGluLeuAlaLeuThrMetArgSerLysLysLeuProIleAsnVal

       ACCACCGGAGAGGAGCAACAAGTAAGCCTGGAATCTGTAGATGTCTACTTTCAAGATGTG
 901   ---------+---------+---------+---------+---------+---------+   960
       ThrThrGlyGluGluGlnGlnValSerLeuGluSerValAspValTyrPheGlnAspVal

       TTTGGAACCATGTGGTGCCACCATGCAGAAATGCAAAACCCCGTGTACCTGATACCAGAA
 961   ---------+---------+---------+---------+---------+---------+   1020
       PheGlyThrMetTrpCysHisHisAlaGluMetGlnAsnProValTyrLeuIleProGlu

       ACAGTGCCATACATAAAGTGGGATAACTGTAATTCTACCAATATAACGGCAGTAGTGAGG
1021   ---------+---------+---------+---------+---------+---------+   1080
```

- 2 -

```
     ThrValProTyrIleLysTrpAspAsnCysAsnSerThrAsnIleThrAlaValValArg

     GCACAGGGGCTGGATGTCACGCTACCCTTAAGTTTGCCAACGTCAGCTCAAGACTCGAAT
1081 ---------+---------+---------+---------+---------+---------+ 1140
     AlaGlnGlyLeuAspValThrLeuProLeuSerLeuProThrSerAlaGlnAspSerAsn

     TTCAGCGTAAAAACAGAAATGCTCGGTAATGAGATAGATATTGAGTGTATTATGGAGGAT
1141 ---------+---------+---------+---------+---------+---------+ 1200
     PheSerValLysThrGluMetLeuGlyAsnGluIleAspIleGluCysIleMetGluAsp
                                                   PstI
     GGCGAAATTTCACAAGTTCTGCCCGGAGACAACAAATTTAACATCACCTGCAGTGGATAC
1201 ---------+---------+---------+---------+---------+---------+ 1260
     GlyGluIleSerGlnValLeuProGlyAspAsnLysPheAsnIleThrCysSerGlyTyr
                               EcoRI
     GAGAGCCATGTTCCCAGCGGCGGAATTCTCACATCAACGAGTCCCGTGGCCACCCCAATA
1261 ---------+---------+---------+---------+---------+---------+ 1320
     GluSerHisValProSerGlyGlyIleLeuThrSerThrSerProValAlaThrProIle

     CCTGGTACAGGGTATGCATACAGCCTGCGTCTGACACCACGTCCAGTGTCACGATTTCTT
1321 ---------+---------+---------+---------+---------+---------+ 1380
     ProGlyThrGlyTyrAlaTyrSerLeuArgLeuThrProArgProValSerArgPheLeu

     GGCAATAACAGTATCCTGTACGTGTTTTACTCTGGGAATGGACCGAAGGCGAGCGGGGGA
1381 ---------+---------+---------+---------+---------+---------+ 1440
     GlyAsnAsnSerIleLeuTyrValPheTyrSerGlyAsnGlyProLysAlaSerGlyGly

     GATTACTGCATTCAGTCCAACATTGTGTTCTCTGATGAGATTCCAGCTTCACAGGACATG
1441 ---------+---------+---------+---------+---------+---------+ 1500
     AspTyrCysIleGlnSerAsnIleValPheSerAspGluIleProAlaSerGlnAspMet

     CCGACAAACACCACAGACATCACATATGTGGGTGACAATGCTACCTATTCAGTGCCAATG
1501 ---------+---------+---------+---------+---------+---------+ 1560
     ProThrAsnThrThrAspIleThrTyrValGlyAspAsnAlaThrTyrSerValProMet

     GTCACTTCTGAGGACGCAAACTCGCCAAATGTTACAGTGACTGCCTTTTGGGCCTGGCCA
1561 ---------+---------+---------+---------+---------+---------+ 1620
     ValThrSerGluAspAlaAsnSerProAsnValThrValThrAlaPheTrpAlaTrpPro

     AACAACACTGAAACTGACTTTAAGTGCAAATGGACTCTCACCTCGGGGACACCTTCGGGT
1621 ---------+---------+---------+---------+---------+---------+ 1680
     AsnAsnThrGluThrAspPheLysCysLysTrpThrLeuThrSerGlyThrProSerGly

     TGTGAAAATATTTCTGGTGCATTTGCGAGCAATCGGACATTTGACATTACTGTCTCGGGT
1681 ---------+---------+---------+---------+---------+---------+ 1740
     CysGluAsnIleSerGlyAlaPheAlaSerAsnArgThrPheAspIleThrValSerGly

     CTTGGCACGGCCCCCAAGACACTCATTATCACACGAACGGCTACCAATGCCACCACAACA
1741 ---------+---------+---------+---------+---------+---------+ 1800
     LeuGlyThrAlaProLysThrLeuIleIleThrArgThrAlaThrAsnAlaThrThrThr

     ACCCACAAGGTTATATTCTCCAAGGCACCCGAGAGCACCACCACCTCCCCTACCTTGAAT
1801 ---------+---------+---------+---------+---------+---------+ 1860
     ThrHisLysValIlePheSerLysAlaProGluSerThrThrThrSerProThrLeuAsn

     ACAACTGGATTTGCTGATCCCAATACAACGACAGGTCTACCCAGCTCTACTCACGTGCCT
1861 ---------+---------+---------+---------+---------+---------+ 1920
     ThrThrGlyPheAlaAspProAsnThrThrThrGlyLeuProSerSerThrHisValPro

     ACCAACCTCACCGCACCTGCAAGCACAGGCCCCACTGTATCCACCGCGGATGTCACCAGC
1921 ---------+---------+---------+---------+---------+---------+ 1980
     ThrAsnLeuThrAlaProAlaSerThrGlyProThrValSerThrAlaAspValThrSer

     CCAACACCAGCCGGCACAACGTCAGGCGCATCACCGGTGACACCAAGTCCATCTCCATGG
```

- 3 -

```
1981  ---------+---------+---------+---------+---------+---------+  2040
      ProThrProAlaGlyThrThrSerGlyAlaSerProValThrProSerProSerProTrp
                    )(-----
      GACAACGGCACAGAAAGTAAGGCCCCGACATGACCAGCTCCACCTCACCAGTGACTACC
2041  ---------+---------+---------+---------+---------+---------+  2100
      AspAsnGlyThrGluSerLysAlaProAspMetThrSerSerThrSerProValThrThr

      CCAACCCCAAATGCCACCAGCCCCACCCCAGCAGTGACTACCCCAACCCCAAATGCCACC
2101  ---------+---------+---------+---------+---------+---------+  2160
      ProThrProAsnAlaThrSerProThrProAlaValThrThrProThrProAsnAlaThr

      AGCCCCACCCCAGCAGTGACTACCCCAACCCCAAATGCCACCAGCCCCACCTTGGGAAAA
2161  ---------+---------+---------+---------+---------+---------+  2220
      SerProThrProAlaValThrThrProThrProAsnAlaThrSerProThrLeuGlyLys

      ACAAGTCCTACCTCAGCAGTGACTACCCCAACCCCAAATGCCACCAGCCCCACCTTGGGA
2221  ---------+---------+---------+---------+---------+---------+  2280
      ThrSerProThrSerAlaValThrThrProThrProAsnAlaThrSerProThrLeuGly

      AAAACAAGCCCCACCTCAGCAGTGACTACCCCAACCCCAAATGCCACCAGCCCCACCTTG
2281  ---------+---------+---------+---------+---------+---------+  2340
      LysThrSerProThrSerAlaValThrThrProThrProAsnAlaThrSerProThrLeu

      GGAAAAACAAGCCCCACCTCAGCAGTGACTACCCCAACCCCAAATGCCACCGGCCCTACT
2341  ---------+---------+---------+---------+---------+---------+  2400
      GlyLysThrSerProThrSerAlaValThrThrProThrProAsnAlaThrGlyProThr

      GTGGGAGAAACAAGTCCACAGGCAAATGCCACCAACCACACCTTAGGAGGAACAAGTCCC
2401  ---------+---------+---------+---------+---------+---------+  2460
      ValGlyGluThrSerProGlnAlaAsnAlaThrAsnHisThrLeuGlyGlyThrSerPro

      ACCCCAGTAGTTACCAGCCAACCAAAAAATGCAACCAGTGCTGTTACCACAGGCCAACAT
2461  ---------+---------+---------+---------+---------+---------+  2520
      ThrProValValThrSerGlnProLysAsnAlaThrSerAlaValThrThrGlyGlnHis

      AACATAACTTCAAGTTCAACCTCTTCCATGTCACTGAGACCCAGTTCAAACCCAGAGACA
2521  ---------+---------+---------+---------+---------+---------+  2580
      AsnIleThrSerSerSerThrSerSerMetSerLeuArgProSerSerAsnProGluThr

      CTCAGCCCCTCCACCAGTGACAATTCAACGTCACATATGCCTTTACTAACCTCCGCTCAC
2581  ---------+---------+---------+---------+---------+---------+  2640
      LeuSerProSerThrSerAspAsnSerThrSerHisMetProLeuLeuThrSerAlaHis
        ----)(
      CCAACAGGTGGTGAAAATATAACACAGGTGACACCAGCCTCTATCAGCACACATCATGTG
2641  ---------+---------+---------+---------+---------+---------+  2700
      ProThrGlyGlyGluAsnIleThrGlnValThrProAlaSerIleSerThrHisHisVal

      TCCACCAGTTCGCCAGCACCCCGCCCAGGCACCACCAGCCAAGCGTCAGGCCCTGGAAAC
2701  ---------+---------+---------+---------+---------+---------+  2760
      SerThrSerSerProAlaProArgProGlyThrThrSerGlnAlaSerGlyProGlyAsn

      AGTTCCACATCCACAAAACCGGGGGAGGTTAATGTCACCAAAGGCACGCCCCCCCAAAAT
2761  ---------+---------+---------+---------+---------+---------+  2820
      SerSerThrSerThrLysProGlyGluValAsnValThrLysGlyThrProProGlnAsn

      GCAACGTCGCCCCAGGCCCCCAGTGGCCAAAAGACGGCGGTTCCCACGGTCACCTCAACA
2821  ---------+---------+---------+---------+---------+---------+  2880
      AlaThrSerProGlnAlaProSerGlyGlnLysThrAlaValProThrValThrSerThr

      GGTGGAAAGGCCAATTCTACCACCGGTGGAAAGCACACCACAGGACATGGAGCCCGGACA
2881  ---------+---------+---------+---------+---------+---------+  2940
      GlyGlyLysAlaAsnSerThrThrGlyGlyLysHisThrThrGlyHisGlyAlaArgThr
```

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```
        AGTACAGAGCCCACCACAGATTACGGCGGTGATTCAACTACGCCAAGACCGAGATACAAT
2941    ---------+---------+---------+---------+---------+---------+    3000
        SerThrGluProThrThrAspTyrGlyGlyAspSerThrThrProArgProArgTyrAsn

        GCGACCACCTATCTACCTCCCAGCACTTCTAGCAAACTGCGGCCCCGCTGGACTTTTACG
3001    ---------+---------+---------+---------+---------+---------+    3060
        AlaThrThrTyrLeuProProSerThrSerSerLysLeuArgProArgTrpThrPheThr

        AGCCCACCGGTTACCACAGCCCAAGCCACCGTGCCAGTCCCGCCAACGTCCCAGCCCAGA
3061    ---------+---------+---------+---------+---------+---------+    3120
        SerProProValThrThrAlaGlnAlaThrValProValProProThrSerGlnProArg
                               PstI            **********************
        TTCTCAAACCTCTCCATGCTAGTACTGCAGTGGGCCTCTCTGGCTGTGCTGACCCTTCTG
3121    ---------+---------+---------+---------+---------+---------+    3180
        PheSerAsnLeuSerMetLeuValLeuGlnTrpAlaSerLeuAlaValLeuThrLeuLeu
        ************
        CTGCTGCTGGTCATGGCGGACTGCGCCTTTAGGCGTAACTTGTCTACATCCCATACCTAC
3181    ---------+---------+---------+---------+---------+---------+    3240
        LeuLeuLeuValMetAlaAspCysAlaPheArgArgAsnLeuSerThrSerHisThrTyr
                                                  ++++++++
        ACCACCCCACCATATGATGACGCCGAGACCTATGTATAAAGTCAATAAAAATTTATTAAT
3241    ---------+---------+---------+---------+---------+---------+    3300
        ThrThrProProTyrAspAspAlaGluThrTyrValEnd

        CAGAAATTTGCACTTTCTTTGCTTCACGTCCCCGGGAGCGGGAGCGGGCACGTCGGGTGG
3301    ---------+---------+---------+---------+---------+---------+    3360

        CGTTGGGGTCGTTTGATTCTCGTGGTCGTGTTCCCTCACC
3361    ---------+---------+---------+---------+    3400
```

Figure 18

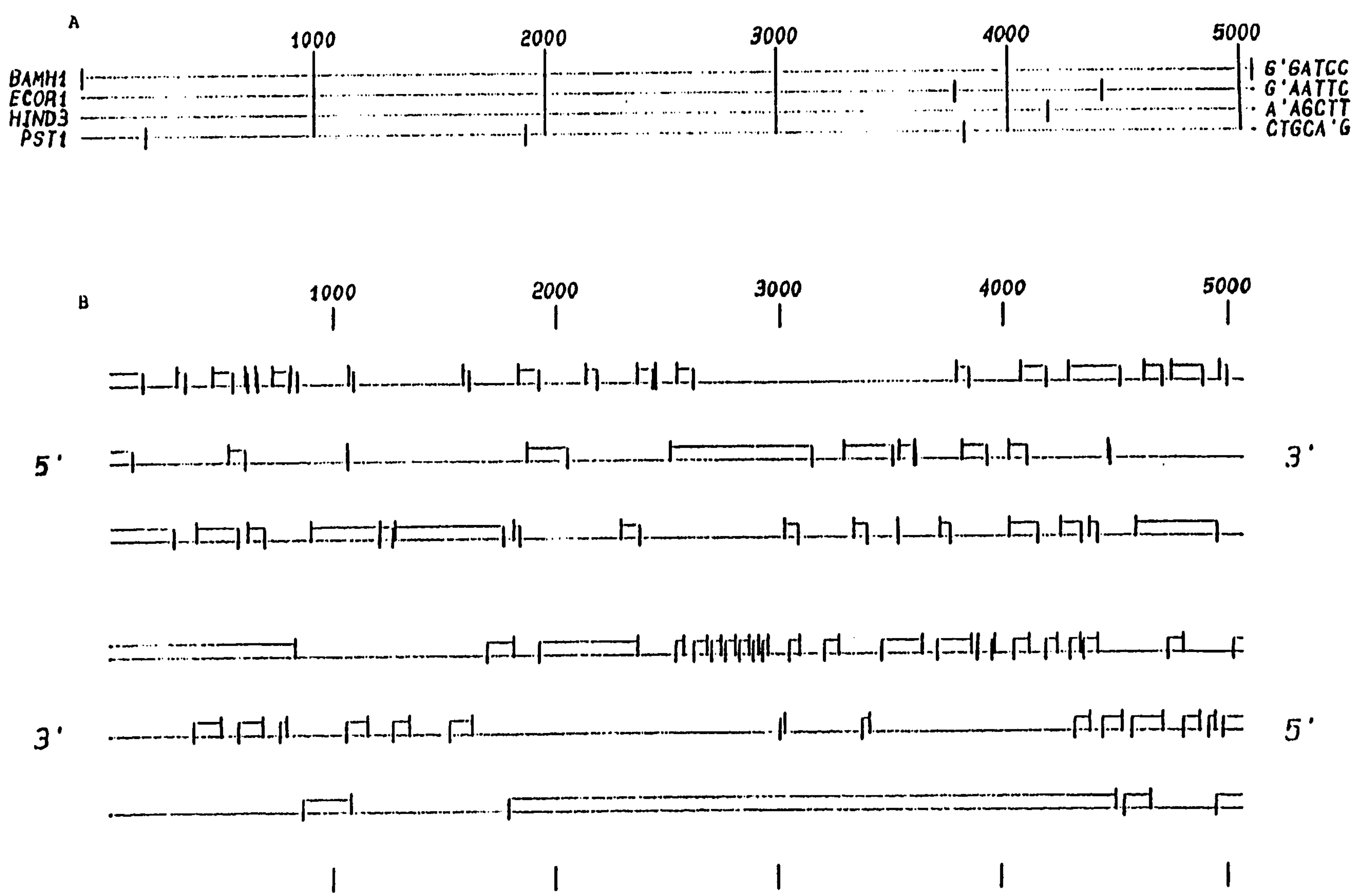
A
1000
2000
3000
4000
5000
BAMH1
ECOR1
HIND3
PST1
G'GATCC
G'AATTC
A'AGCTT
CTGCA'G
B
1000
2000
3000
4000
5000
5'
3'
3'
5'

Figure 19

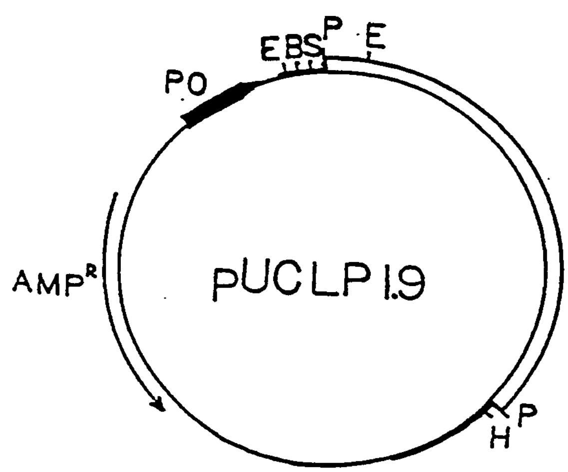

Figure 20

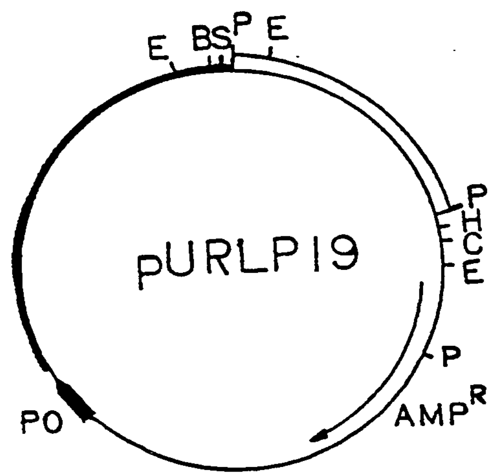

Figure 21 -1-

```
      ACCATGATTACGGATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGC
  1   ---------+---------+---------+---------+---------+---------+   60
         MetIleThrAspSerLeuAlaValValLeuGlnArgArgAspTrpGluAsnProGly

      GTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAA
 61   ---------+---------+---------+---------+---------+---------+  120
      ValThrGlnLeuAsnArgLeuAlaAlaHisProProPheAlaSerTrpArgAsnSerGlu

      GAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTT
121   ---------+---------+---------+---------+---------+---------+  180
      GluAlaArgThrAspArgProSerGlnGlnLeuArgSerLeuAsnGlyGluTrpArgPhe

      GCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTGAG
181   ---------+---------+---------+---------+---------+---------+  240
      AlaTrpPheProAlaProGluAlaValProGluSerTrpLeuGluCysAspLeuProGlu

      GCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCGCCCATCTAC
241   ---------+---------+---------+---------+---------+---------+  300
      AlaAspThrValValValProSerAsnTrpGlnMetHisGlyTyrAspAlaProIleTyr

      ACCAACGTAACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCCACGGAGAATCCGACG
301   ---------+---------+---------+---------+---------+---------+  360
      ThrAsnValThrTyrProIleThrValAsnProProPheValProThrGluAsnProThr

      GGTTGTTACTCGCTCACATTTAATGTTGATGAAAGCTGGCTACAGGAAGGCCAGACGCGA
361   ---------+---------+---------+---------+---------+---------+  420
      GlyCysTyrSerLeuThrPheAsnValAspGluSerTrpLeuGlnGluGlyGlnThrArg

      ATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGCGCTGGGTCGGT
421   ---------+---------+---------+---------+---------+---------+  480
      IleIlePheAspGlyValAsnSerAlaPheHisLeuTrpCysAsnGlyArgTrpValGly

      TACGGCCAGGACAGTCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTACGCGCCGGA
481   ---------+---------+---------+---------+---------+---------+  540
      TyrGlyGlnAspSerArgLeuProSerGluPheAspLeuSerAlaPheLeuArgAlaGly

      GAAAACCGCCTCGCGGTGATGGTGCTGCGTTGGAGTGACGGCAGTTATCTGGAAGATCAG
541   ---------+---------+---------+---------+---------+---------+  600
      GluAsnArgLeuAlaValMetValLeuArgTrpSerAspGlySerTyrLeuGluAspGln

      GATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCATAAACCGACTACA
601   ---------+---------+---------+---------+---------+---------+  660
      AspMetTrpArgMetSerGlyIlePheArgAspValSerLeuLeuHisLysProThrThr

      CAAATCAGCGATTTCCATGTTGCCACTCGCTTTAATGATGATTTCAGCCGCGCTGTACTG
661   ---------+---------+---------+---------+---------+---------+  720
      GlnIleSerAspPheHisValAlaThrArgPheAsnAspAspPheSerArgAlaValLeu

      GAGGCTGAAGTTCAGATGTGCGGCGAGTTGCGTGACTACCTACGGGTAACAGTTTCTTTA
721   ---------+---------+---------+---------+---------+---------+  780
      GluAlaGluValGlnMetCysGlyGluLeuArgAspTyrLeuArgValThrValSerLeu

      TGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAATTATCGAT
781   ---------+---------+---------+---------+---------+---------+  840
      TrpGlnGlyGluThrGlnValAlaSerGlyThrAlaProPheGlyGlyGluIleIleAsp

      GAGCGTGGTGGTTATGCCGATCGCGTCACACTACGTCTGAACGTCGAAAACCCGAAACTG
841   ---------+---------+---------+---------+---------+---------+  900
      GluArgGlyGlyTyrAlaAspArgValThrLeuArgLeuAsnValGluAsnProLysLeu

      TGGAGCGCCGAAATCCCGAATCTCTATCGTGCGGTGGTTGAACTGCACACCGCCGACGGC
```

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```
901   ---------+---------+---------+---------+---------+---------+  960
      TrpSerAlaGluIleProAsnLeuTyrArgAlaValValGluLeuHisThrAlaAspGly

      ACGCTGATTGAAGCAGAAGCCTGCGATGTCGGTTTCCGCGAGGTGCGGATTGAAAATGGT
961   ---------+---------+---------+---------+---------+---------+  1020
      ThrLeuIleGluAlaGluAlaCysAspValGlyPheArgGluValArgIleGluAsnGly

      CTGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCGAGGCGTTAACCGTCACGAGCATCAT
1021  ---------+---------+---------+---------+---------+---------+  1080
      LeuLeuLeuLeuAsnGlyLysProLeuLeuIleArgGlyValAsnArgHisGluHisHis

      CCTCTGCATGGTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCCTGCTGATGAAG
1081  ---------+---------+---------+---------+---------+---------+  1140
      ProLeuHisGlyGlnValMetAspGluGlnThrMetValGlnAspIleLeuLeuMetLys

      CAGAACAACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCGCTGTGGTACACG
1141  ---------+---------+---------+---------+---------+---------+  1200
      GlnAsnAsnPheAsnAlaValArgCysSerHisTyrProAsnHisProLeuTrpTyrThr

      CTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACCCACGGCATG
1201  ---------+---------+---------+---------+---------+---------+  1260
      LeuCysAspArgTyrGlyLeuTyrValValAspGluAlaAsnIleGluThrHisGlyMet

      GTGCCAATGAATCGTCTGACCGATGATCCGCGCTGGCTACCGGCGATGAGCGAACGCGTA
1261  ---------+---------+---------+---------+---------+---------+  1320
      ValProMetAsnArgLeuThrAspAspProArgTrpLeuProAlaMetSerGluArgVal

      ACGCGAATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGGAAT
1321  ---------+---------+---------+---------+---------+---------+  1380
      ThrArgMetValGlnArgAspArgAsnHisProSerValIleIleTrpSerLeuGlyAsn

      GAATCAGGCCACGGCGCTAATCACGACGCGCTGTATCGCTGGATCAAATCTGTCGATCCT
1381  ---------+---------+---------+---------+---------+---------+  1440
      GluSerGlyHisGlyAlaAsnHisAspAlaLeuTyrArgTrpIleLysSerValAspPro

      TCCCGCCCGGTGCAGTATGAAGGCGGCGGAGCCGACACCACGGCCACCGATATTATTTGC
1441  ---------+---------+---------+---------+---------+---------+  1500
      SerArgProValGlnTyrGluGlyGlyGlyAlaAspThrThrAlaThrAspIleIleCys

      CCGATGTACGCGCGCGTGGATGAAGACCAGCCCTTCCCGGCTGTGCCGAAATGGTCCATC
1501  ---------+---------+---------+---------+---------+---------+  1560
      ProMetTyrAlaArgValAspGluAspGlnProPheProAlaValProLysTrpSerIle

      AAAAAATGGCTTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCAC
1561  ---------+---------+---------+---------+---------+---------+  1620
      LysLysTrpLeuSerLeuProGlyGluThrArgProLeuIleLeuCysGluTyrAlaHis

      GCGATGGGTAACAGTCTTGGCGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCC
1621  ---------+---------+---------+---------+---------+---------+  1680
      AlaMetGlyAsnSerLeuGlyGlyPheAlaLysTyrTrpGlnAlaPheArgGlnTyrPro

      CGTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTAAATATGATGAA
1681  ---------+---------+---------+---------+---------+---------+  1740
      ArgLeuGlnGlyGlyPheValTrpAspTrpValAspGlnSerLeuIleLysTyrAspGlu

      AACGGCAACCCGTGGTCGGCTTACGGCGGTGATTTTGGCGATACGCCGAACGATCGCCAG
1741  ---------+---------+---------+---------+---------+---------+  1800
      AsnGlyAsnProTrpSerAlaTyrGlyGlyAspPheGlyAspThrProAsnAspArgGln

      TTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCAGCGCTGACGGAAGCA
1801  ---------+---------+---------+---------+---------+---------+  1860
      PheCysMetAsnGlyLeuValPheAlaAspArgThrProHisProAlaLeuThrGluAla
```

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```
     AAACACCAGCAGCAGTTTTTCCAGTTCCGTTTATCCGGGCAAACCATCGAAGTGACCAGC
1861 ---------+---------+---------+---------+---------+---------+ 1920
     LysHisGlnGlnGlnPhePheGlnPheArgLeuSerGlyGlnThrIleGluValThrSer

     GAATACCTGTTCCGTCATAGCGATAACGAGCTCCTGCACTGGATGGTGGCGCTGGATGGT
1921 ---------+---------+---------+---------+---------+---------+ 1980
     GluTyrLeuPheArgHisSerAspAsnGluLeuLeuHisTrpMetValAlaLeuAspGly

     AAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTCGCTCCACAAGGTAAACAGTTGATT
1981 ---------+---------+---------+---------+---------+---------+ 2040
     LysProLeuAlaSerGlyGluValProLeuAspValAlaProGlnGlyLysGlnLeuIle

     GAACTGCCTGAACTACCGCAGCCGGAGAGCGCCGGGCAACTCTGGCTCACAGTACGCGTA
2041 ---------+---------+---------+---------+---------+---------+ 2100
     GluLeuProGluLeuProGlnProGluSerAlaGlyGlnLeuTrpLeuThrValArgVal

     GTGCAACCGAACGCGACCGCATGGTCAGAAGCCGGGCACATCAGCGCCTGGCAGCAGTGG
2101 ---------+---------+---------+---------+---------+---------+ 2160
     ValGlnProAsnAlaThrAlaTrpSerGluAlaGlyHisIleSerAlaTrpGlnGlnTrp

     CGTCTGGCGGAAAACCTCAGTGTGACGCTCCCCGCCGCGTCCCACGCCATCCCGCATCTG
2161 ---------+---------+---------+---------+---------+---------+ 2220
     ArgLeuAlaGluAsnLeuSerValThrLeuProAlaAlaSerHisAlaIleProHisLeu

     ACCACCAGCGAAATGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAATTTAACCGC
2221 ---------+---------+---------+---------+---------+---------+ 2280
     ThrThrSerGluMetAspPheCysIleGluLeuGlyAsnLysArgTrpGlnPheAsnArg

     CAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATAAAAAACAACTGCTGACGCCGCTG
2281 ---------+---------+---------+---------+---------+---------+ 2340
     GlnSerGlyPheLeuSerGlnMetTrpIleGlyAspLysLysGlnLeuLeuThrProLeu

     CGCGATCAGTTCACCCGTGCACCGCTGGATAACGACATTGGCGTAAGTGAAGCGACCCGC
2341 ---------+---------+---------+---------+---------+---------+ 2400
     ArgAspGlnPheThrArgAlaProLeuAspAsnAspIleGlyValSerGluAlaThrArg

     ATTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCCGAAGCA
2401 ---------+---------+---------+---------+---------+---------+ 2460
     IleAspProAsnAlaTrpValGluArgTrpLysAlaAlaGlyHisTyrGlnAlaGluAla

     GCGTTGTTGCAGTGCACGGCAGATACACTTGCTGATGCGGTGCTGATTACGACCGCTCAC
2461 ---------+---------+---------+---------+---------+---------+ 2520
     AlaLeuLeuGlnCysThrAlaAspThrLeuAlaAspAlaValLeuIleThrThrAlaHis

     GCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGATGGT
2521 ---------+---------+---------+---------+---------+---------+ 2580
     AlaTrpGlnHisGlnGlyLysThrLeuPheIleSerArgLysThrTyrArgIleAspGly

     AGTGGTCAAATGGCGATTACCGTTGATGTTGAAGTGGCGAGCGATACACCGCATCCGGCG
2581 ---------+---------+---------+---------+---------+---------+ 2640
     SerGlyGlnMetAlaIleThrValAspValGluValAlaSerAspThrProHisProAla

     CGGATTGGCCTGAACTGCCAGCTGGCGCAGGTAGCAGAGCGGGTAAACTGGCTCGGATTA
2641 ---------+---------+---------+---------+---------+---------+ 2700
     ArgIleGlyLeuAsnCysGlnLeuAlaGlnValAlaGluArgValAsnTrpLeuGlyLeu

     GGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCCGCCTGTTTTGACCGCTGGGATCTG
2701 ---------+---------+---------+---------+---------+---------+ 2760
     GlyProGlnGluAsnTyrProAspArgLeuThrAlaAlaCysPheAspArgTrpAspLeu

     CCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGCTGCGGG
2761 ---------+---------+---------+---------+---------+---------+ 2820
     ProLeuSerAspMetTyrThrProTyrValPheProSerGluAsnGlyLeuArgCysGly
```

-4-

```
      ACGCGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATCAGC
2821  ---------+---------+---------+---------+---------+---------+  2880
      ThrArgGluLeuAsnTyrGlyProHisGlnTrpArgGlyAspPheGlnPheAsnIleSer

      CGCTACAGTCAACAGCAACTGATGGAAACCAGCCATCGCCATCTGCTGCACGCGGAAGAA
2881  ---------+---------+---------+---------+---------+---------+  2940
      ArgTyrSerGlnGlnGlnLeuMetGluThrSerHisArgHisLeuLeuHisAlaGluGlu

      GGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGACTCCTGGAGC
2941  ---------+---------+---------+---------+---------+---------+  3000
      GlyThrTrpLeuAsnIleAspGlyPheHisMetGlyIleGlyGlyAspAspSerTrpSer

      CCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGTCTGG
3001  ---------+---------+---------+---------+---------+---------+  3060
      ProSerValSerAlaGluPheGlnLeuSerAlaGlyArgTyrHisTyrGlnLeuValTrp

      TGTCAAAAAggggatccgtcgacctgcaGTGGATACGAGAGCCATGTTCCCAGCGGCGGA
3061  ---------+---------+---------+---------+---------+---------+  3120
      CysGlnLysGlyAspProSerThrCysSerGlyTyrGluSerHisValProSerGlyGly

      ATTCTCACATCAACGAGTCCCGTGGCCACCCCAATACCTGGTACAGGGTATGCATACAGC
3121  ---------+---------+---------+---------+---------+---------+  3180
      IleLeuThrSerThrSerProValAlaThrProIleProGlyThrGlyTyrAlaTyrSer

      CTGCGTCTGACACCACGTCCAGTGTCACGATTTCTTGGCAATAACAGTATCCTGTACGTG
3181  ---------+---------+---------+---------+---------+---------+  3240
      LeuArgLeuThrProArgProValSerArgPheLeuGlyAsnAsnSerIleLeuTyrVal

      TTTTACTCTGGGAATGGACCGAAGGCGAGCGGGGGAGATTACTGCATTCAGTCCAACATT
3241  ---------+---------+---------+---------+---------+---------+  3300
      PheTyrSerGlyAsnGlyProLysAlaSerGlyGlyAspTyrCysIleGlnSerAsnIle

      GTGTTCTCTGATGAGATTCCAGCTTCACAGGACATGCCGACAAACACCACAGACATCACA
3301  ---------+---------+---------+---------+---------+---------+  3360
      ValPheSerAspGluIleProAlaSerGlnAspMetProThrAsnThrThrAspIleThr

      TATGTGGGTGACAATGCTACCTATTCAGTGCCAATGGTCACTTCTGAGGACGCAAACTCG
3361  ---------+---------+---------+---------+---------+---------+  3420
      TyrValGlyAspAsnAlaThrTyrSerValProMetValThrSerGluAspAlaAsnSer

      CCAAATGTTACAGTGACTGCCTTTTGGGCCTGGCCAAACAACACTGAAACTGACTTTAAG
3421  ---------+---------+---------+---------+---------+---------+  3480
      ProAsnValThrValThrAlaPheTrpAlaTrpProAsnAsnThrGluThrAspPheLys

      TGCAAATGGACTCTCACCTCGGGGACACCTTCGGGTTGTGAAAATATTTCTGGTGCATTT
3481  ---------+---------+---------+---------+---------+---------+  3540
      CysLysTrpThrLeuThrSerGlyThrProSerGlyCysGluAsnIleSerGlyAlaPhe

      GCGAGCAATCGGACATTTGACATTACTGTCTCGGGTCTTGGCACGGCCCCCAAGACACTC
3541  ---------+---------+---------+---------+---------+---------+  3600
      AlaSerAsnArgThrPheAspIleThrValSerGlyLeuGlyThrAlaProLysThrLeu

      ATTATCACACGAACGGCTACCAATGCCACCACAACAACCCACAAGGTTATATTCTCCAAG
3601  ---------+---------+---------+---------+---------+---------+  3660
      IleIleThrArgThrAlaThrAsnAlaThrThrThrThrHisLysValIlePheSerLys

      GCACCCGAGAGCACCACCACCTCCCCTACCTTGAATACAACTGGATTTGCTGATCCCAAT
3661  ---------+---------+---------+---------+---------+---------+  3720
      AlaProGluSerThrThrThrSerProThrLeuAsnThrThrGlyPheAlaAspProAsn

      ACAACGACAGGTCTACCCAGCTCTACTCACGTGCCTACCAACCTCACCGCACCTGCAAGC
3721  ---------+---------+---------+---------+---------+---------+  3780
```

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```
     ThrThrThrGlyLeuProSerSerThrHisValProThrAsnLeuThrAlaProAlaSer

     ACAGGCCCCACTGTATCCACCGCGGATGTCACCAGCCCAACACCAGCCGGCACAACGTCA
3781 ---------+---------+---------+---------+---------+---------+ 3840
     ThrGlyProThrValSerThrAlaAspValThrSerProThrProAlaGlyThrThrSer

     GGCGCATCACCGGTGACACCAAGTCCATCTCCATGGGACAACGGCACAGAAAGTAAGGCC
3841 ---------+---------+---------+---------+---------+---------+ 3900
     GlyAlaSerProValThrProSerProSerProTrpAspAsnGlyThrGluSerLysAla

     CCCGACATGACCAGCTCCACCTCACCAGTGACTACCCCAACCCCAAATGCCACCAGCCCC
3901 ---------+---------+---------+---------+---------+---------+ 3960
     ProAspMetThrSerSerThrSerProValThrThrProThrProAsnAlaThrSerPro

     ACCCCAGCAGTGACTACCCCAACCCCAAATGCCACCAGCCCCACCCCAGCAGTGACTACC
3961 ---------+---------+---------+---------+---------+---------+ 4020
     ThrProAlaValThrThrProThrProAsnAlaThrSerProThrProAlaValThrThr

     CCAACCCCAAATGCCACCAGCCCCACCTTGGGAAAAACAAGTCCTACCTCAGCAGTGACT
4021 ---------+---------+---------+---------+---------+---------+ 4080
     ProThrProAsnAlaThrSerProThrLeuGlyLysThrSerProThrSerAlaValThr

     ACCCCAACCCCAAATGCCACCAGCCCCACCTTGGGAAAAACAAGCCCCACCTCAGCAGTG
4081 ---------+---------+---------+---------+---------+---------+ 4140
     ThrProThrProAsnAlaThrSerProThrLeuGlyLysThrSerProThrSerAlaVal

     ACTACCCCAACCCCAAATGCCACCAGCCCCACCTTGGGAAAAACAAGCCCCACCTCAGCA
4141 ---------+---------+---------+---------+---------+---------+ 4200
     ThrThrProThrProAsnAlaThrSerProThrLeuGlyLysThrSerProThrSerAla

     GTGACTACCCCAACCCCAAATGCCACCGGCCCTACTGTGGGAGAAACAAGTCCACAGGCA
4201 ---------+---------+---------+---------+---------+---------+ 4260
     ValThrThrProThrProAsnAlaThrGlyProThrValGlyGluThrSerProGlnAla

     AATGCCACCAACCACACCTTAGGAGGAACAAGTCCCACCCCAGTAGTTACCAGCCAACCA
4261 ---------+---------+---------+---------+---------+---------+ 4320
     AsnAlaThrAsnHisThrLeuGlyGlyThrSerProThrProValValThrSerGlnPro

     AAAAATGCAACCAGTGCTGTTACCACAGGCCAACATAACATAACTTCAAGTTCAACCTCT
4321 ---------+---------+---------+---------+---------+---------+ 4380
     LysAsnAlaThrSerAlaValThrThrGlyGlnHisAsnIleThrSerSerSerThrSer

     TCCATGTCACTGAGACCCAGTTCAAACCCAGAGACACTCAGCCCCTCCACCAGTGACAAT
4381 ---------+---------+---------+---------+---------+---------+ 4440
     SerMetSerLeuArgProSerSerAsnProGluThrLeuSerProSerThrSerAspAsn

     TCAACGTCACATATGCCTTTACTAACCTCCGCTCACCCAACAGGTGGTGAAAATATAACA
4441 ---------+---------+---------+---------+---------+---------+ 4500
     SerThrSerHisMetProLeuLeuThrSerAlaHisProThrGlyGlyGluAsnIleThr

     CAGGTGACACCAGCCTCTATCAGCACACATCATGTGTCCACCAGTTCGCCAGCACCCCGC
4501 ---------+---------+---------+---------+---------+---------+ 4560
     GlnValThrProAlaSerIleSerThrHisHisValSerThrSerSerProAlaProArg

     CCAGGCACCACCAGCCAAGCGTCAGGCCCTGGAAACAGTTCCACATCCACAAAACCGGGG
4561 ---------+---------+---------+---------+---------+---------+ 4620
     ProGlyThrThrSerGlnAlaSerGlyProGlyAsnSerSerThrSerThrLysProGly

     GAGGTTAATGTCACCAAAGGCACGCCCCCCCAAAATGCAACGTCGCCCCAGGCCCCCAGT
4621 ---------+---------+---------+---------+---------+---------+ 4680
     GluValAsnValThrLysGlyThrProProGlnAsnAlaThrSerProGlnAlaProSer

     GGCCAAAAGACGGCGGTTCCCACGGTCACCTCAACAGGTGGAAAGGCCAATTCTACCACC
```

```
                                                                                    -6-
4681 ---------+---------+---------+---------+---------+---------+ 4740
     GlyGlnLysThrAlaValProThrValThrSerThrGlyGlyLysAlaAsnSerThrThr

     GGTGGAAAGCACACCACAGGACATGGAGCCCGGACAAGTACAGAGCCCACCACAGATTAC
4741 ---------+---------+---------+---------+---------+---------+ 4800
     GlyGlyLysHisThrThrGlyHisGlyAlaArgThrSerThrGluProThrThrAspTyr

     GGCGGTGATTCAACTACGCCAAGACCGAGATACAATGCGACCACCTATCTACCTCCCAGC
4801 ---------+---------+---------+---------+---------+---------+ 4860
     GlyGlyAspSerThrThrProArgProArgTyrAsnAlaThrThrTyrLeuProProSer

     ACTTCTAGCAAACTGCGGCCCCGCTGGACTTTTACGAGCCCACCGGTTACCACAGCCCAA
4861 ---------+---------+---------+---------+---------+---------+ 4920
     ThrSerSerLysLeuArgProArgTrpThrPheThrSerProProValThrThrAlaGln

     GCCACCGTGCCAGTCCCGCCAACGTCCCAGCCCAGATTCTCAAACCTCTCCATGCTAGTA
4921 ---------+---------+---------+---------+---------+---------+ 4980
     AlaThrValProValProProThrSerGlnProArgPheSerAsnLeuSerMetLeuVal

     CTGCAGccaagcttATCGATGATAAGCTGTCAAACATGA
4981 ---------+---------+---------+---------  5019
     LeuGlnProSerLeuSerMetIleSerCysGlnThrEnd
```

Figure 22

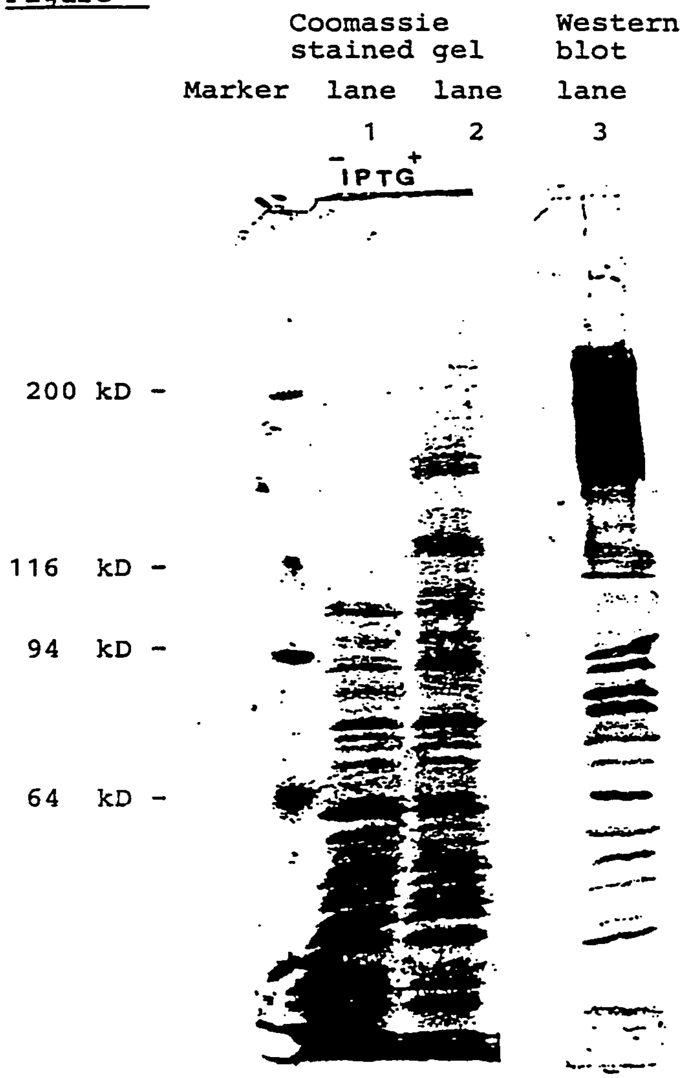

Coomassie stained gel
Western blot
Marker
lane 1
lane 2
lane 3
- IPTG +
200 kD -
116 kD -
94 kD -
64 kD -

Figure 23

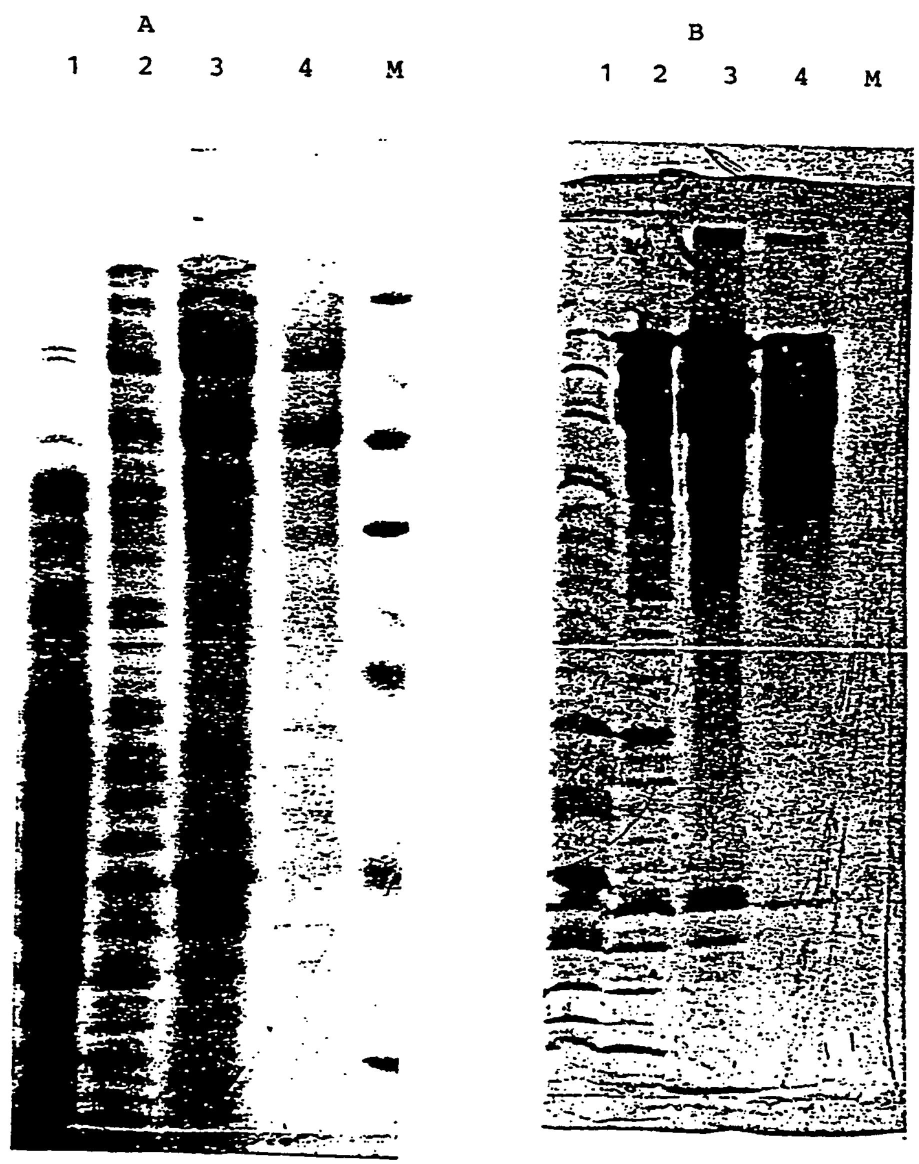
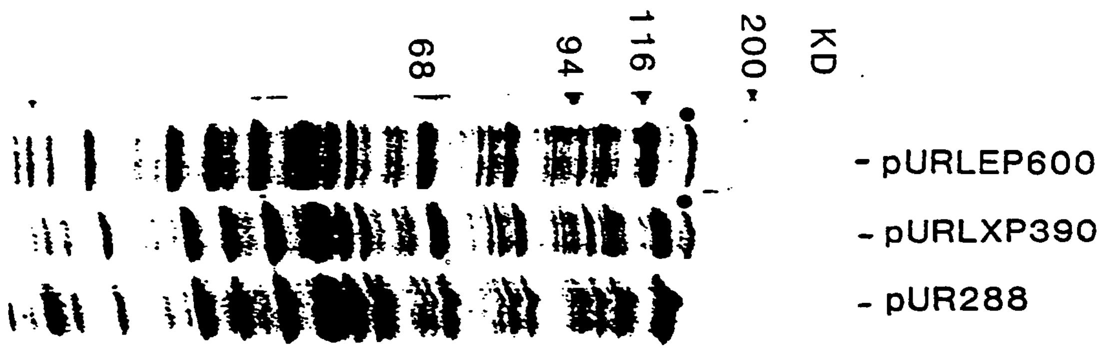


Figure 24

Figure 25:

Expression of gp350-fragments as ß-gal fusion proteins



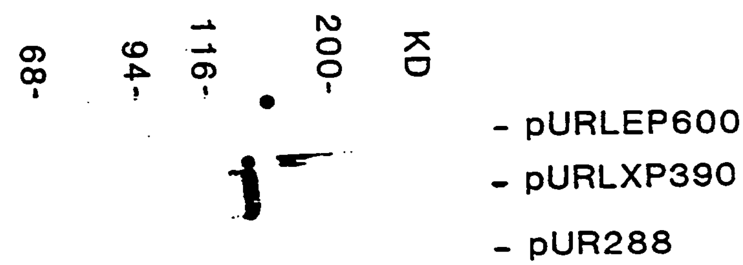



Figure 26:

Expression of proteins

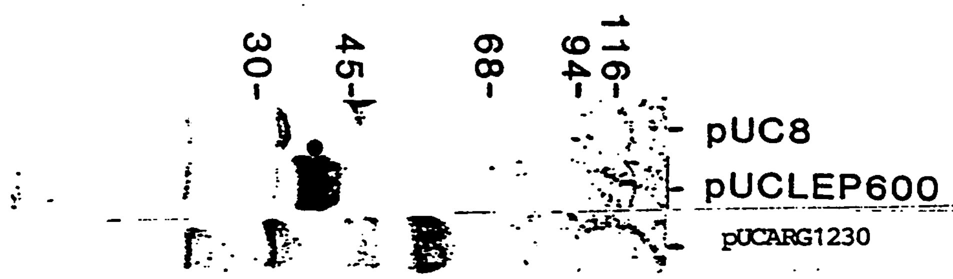
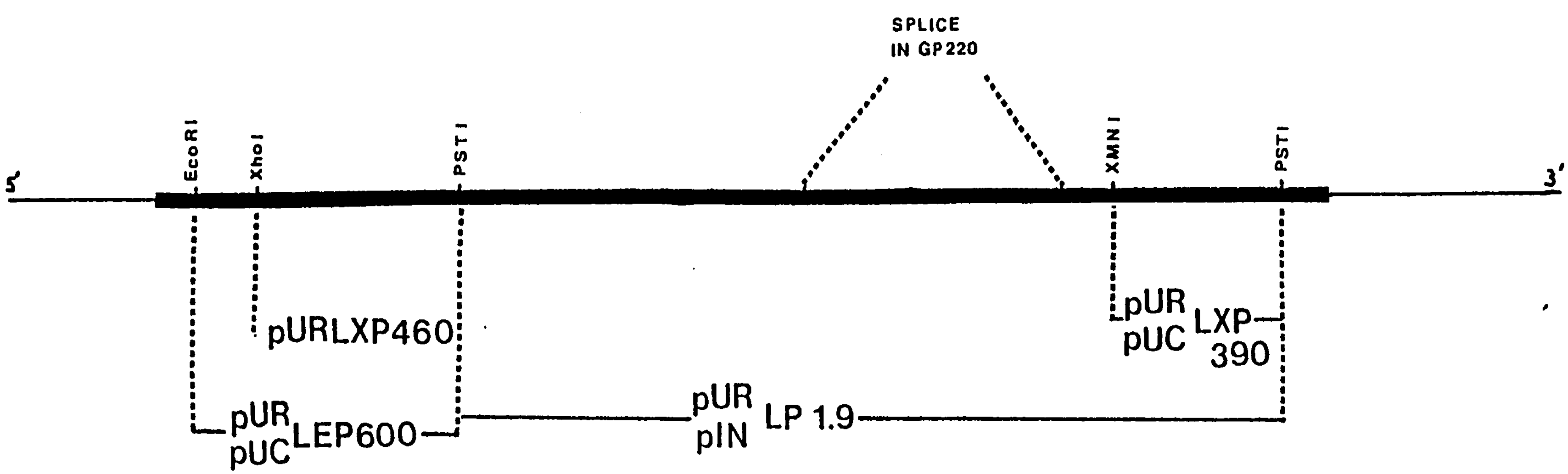


Figure 27: Restriction map of the coding region of gp 250/350



0173254

Figure 28



A: p47

```
         GTACGTTGGCTTCTGCTGCTGCTTGTGATCATGGAAACCACTCAGACTCTCCGCTTTAAG
79869    ---------+---------+---------+---------+---------+---------+ 79928
         CATGCAACCGAAGACGACGACGAACACTAGTACCTTTGGTGAGTCTGAGAGGCGAAATTC

c                                        M  E  T  T  Q  T  L  R  E  K

         ACCAAGGCCCTAGCCGTCCTGTCCAAGTGCTATGACCATGCCCAGACTCATCTCAAGGGA
79929    ---------+---------+---------+---------+---------+---------+ 79988
         TGGTTCCGGGATCGGCAGGACAGGTTCACGATACTGGTACGGGTCTGAGTAGAGTTCCCT

c        T  K  A  L  A  V  L  S  K  C  Y  D  H  A  Q  T  H  L  K  G

         GGAGTGCTGCAGGTAAACCTTCTGTCTGTAAACTATGGAGGCCCCCGGCTGGCCGCCGTG
79989    ---------+---------+---------+---------+---------+---------+ 80048
         CCTCACGACGTCCATTTGGAAGACAGACATTTGATACCTCCGGGGGCCGACCGGCGGCAC

c        G  V  L  Q  V  N  L  L  S  V  N  Y  G  G  P  R  L  A  A  V

         GCCAACGCAGGCACGGCCGGGCTAATCAGCTTCGAGGTCTCCCCTGACGCTGTGGCCGAG
80049    ---------+---------+---------+---------+---------+---------+ 80108
         CGGTTGCGTCCGTGCCGGCCCGATTAGTCGAAGCTCCAGAGGGGACTGCGACACCGGCTC

c        A  N  A  G  T  A  G  L  I  S  F  E  V  S  P  D  A  V  A  E

         TGGCAGAATCACCAGAGCCCAGAGGAGGCCCCGGCCGCCGTGTCATTTAGAAACCTTGCC
80109    ---------+---------+---------+---------+---------+---------+ 80168
         ACCGTCTTAGTGGTCTCGGGTCTCCTCCGGGGCCGGCGGCACAGTAAATCTTTGGAACGG

c        W  Q  N  H  Q  S  P  E  E  A  P  A  A  V  S  F  R  N  L  A

         TACGGGCGCACCTGTGTCCTGGGCAAGGAGCTGTTTGGCTCGGCTGTGGAGCAGGCTTCC
80169    ---------+---------+---------+---------+---------+---------+ 80228
         ATGCCCGCGTGGACACAGGACCCGTTCCTCGACAAACCGAGCCGACACCTCGTCCGAAGG

c        Y  G  R  T  C  V  L  G  K  E  L  F  G  S  A  V  E  Q  A  S

         CTGCAATTTTACAAGCGGCCACAAGGGGGTTCCCGGCCTGAATTTGTTAAGCTCACTATG
80229    ---------+---------+---------+---------+---------+---------+ 80288
         GACGTTAAAATGTTCGCCGGTGTTCCCCCAAGGGCCGGACTTAAACAATTCGAGTGATAC

c        L  Q  F  Y  K  R  P  Q  G  G  S  R  P  E  F  V  K  L  T  M

         GAATATGATGATAAGGTGTCCAAGAGCCACCACACCTGCGCCCTGATGCCCTATATGCCC
80289    ---------+---------+---------+---------+---------+---------+ 80348
         CTTATACTACTATTCCACAGGTTCTCGGTGGTGTGGACGCGGGACTACGGGATATACGGG

c        E  Y  D  D  K  V  S  K  S  H  H  T  C  A  L  M  P  Y  M  P

         CCGGCCAGCGACAGGCTGAGGAACGAGCAGATGATTGGGCAGGTGCTGTTGATGCCCAAG
80349    ---------+---------+---------+---------+---------+---------+ 80408
         GGCCGGTCGCTGTCCGACTCCTTGCTCGTCTACTAACCCGTCCACGACAACTACGGGTTC

c        P  A  S  D  R  L  R  N  E  Q  M  I  G  Q  V  L  L  M  P  K

         ACGGCTTCCTCGTTGCAGAAGTGGGCACGCCAGCAAGGCTCAGGCGGCGTTAAGGTGACA
80409    ---------+---------+---------+---------+---------+---------+ 80468
         TGCCGAAGGAGCAACGTCTTCACCCGTGCGGTCGTTCCGAGTCCGCCGCAATTCCACTGT
```

- 2 -

```
c        T  A  S  S  L  Q  K  W  A  R  Q  Q  G  S  G  G  V  K  V  T

         CTCAATCCGGATCTCTACGTCACCACGTATACTTCTGGGGAGGCCTGCCTCACCCTAGAC
   80469 -+---------+---------+---------+---------+---------+--------- 80528
         GAGTTAGGCCTAGAGATGCAGTGGTGCATATGAAGACCCCTCCGGACGGAGTGGGATCTG

c        L  N  P  D  L  Y  V  I  T  Y  T  S  G  E  A  C  L  I  L  D

         TACAAGCCTCTGAGTGTGGGGCCATACGAGGCCTTCACTGGCCCTGTGGCCAAGGCTCAG
   80529 -+---------+---------+---------+---------+---------+--------- 80588
         ATGTTCGGAGACTCACACCCCGGTATGCTCCGGAAGTGACCGGGACACCGGTTCCGAGTC

c        Y  K  P  L  S  V  G  P  Y  E  A  E  T  G  P  V  A  K  A  Q

         GACGTGGGGGCCGTTGAGGCCCACGTTGTCTGCTCGGTAGCAGCGGACTCGCTGGCGGCG
   80589 -+---------+---------+---------+---------+---------+--------- 80648
         CTGCACCCCCGGCAACTCCGGGTGCAACAGACGAGCCATCGTCGCCTGAGCGACCGCCGC

c        D  V  G  A  V  E  A  H  V  V  C  S  V  A  A  D  S  L  A  A

         GCGCTTAGCCTCTGCCGCATTCCGGCCGTTAGCGTGCCAATCTTGAGGTTTTACAGGTCT
   80649 -+---------+---------+---------+---------+---------+--------- 80708
         CGCGAATCGGAGACGGCGTAAGGCCGGCAATCGCACGGTTAGAACTCCAAAATGTCCAGA

c        A  L  S  L  C  R  I  P  A  V  S  V  P  I  L  R  F  Y  R  S

         GGCATCATAGCTGTGGTGGCCGGCCTGCTGACGTCAGCGGGGGACCTGCCGTTGGATCTT
   80709 -+---------+---------+---------+---------+---------+--------- 80768
         CCGTAGTATCGACACCACCGGCCGGACGACTGCAGTCGCCCCCTGGACGGCAACCTAGAA

c        G  I  I  A  V  V  A  G  L  L  T  S  A  G  D  L  P  L  D  L

         AGTGTTATTTTATTTAACCACGCCTCCGAAGAGGCGGCCGCCAGTACGGCCTCTGAGCCA
   80769 -+---------+---------+---------+---------+---------+--------- 80828
         TCACAATAAAATAAATTGGTGCGGAGGCTTCTCCGCCGGCGGTCATGCCGGAGACTCGGT

c        S  V  I  L  F  N  H  A  S  E  E  A  A  A  S  T  A  S  E  P

         GAAGATAAAAGTCCCCGGGTGCAACCACTGGGCACAGGACTCCAACAACGCCCCAGACAT
   80829 -+---------+---------+---------+---------+---------+--------- 80888
         CTTCTATTTTCAGGGGCCCACGTTGGTGACCCGTGTCCTGAGGTTGTTGCGGGGTCTGTA

c        E  D  K  S  P  R  V  Q  P  L  G  T  G  L  Q  Q  R  P  R  H

         ACGGTCAGTCCATCTCCTTCACCTCCGCCACCTCCTAGGACCCCTACTTGGGAGAGTCCG
   80889 -+---------+---------+---------+---------+---------+--------- 80948
         TGCCAGTCAGGTAGAGGAAGTGGAGGCGGTGGAGGATCCTGGGGATGAACCCTCTCAGGC

c        T  V  S  P  S  P  S  P  P  P  P  P  R  T  P  T  W  E  S  P

         GCAAGGCCAGAGACACCCTCGCCTGCCATTCCCAGCCACTCCAGCAACACCGCACTGGAG
   80949 -+---------+---------+---------+---------+---------+--------- 81008
         CGTTCCGGTCTCTGTGGGAGCGGACGGTAAGGGTCGGTGAGGTCGTTGTGGCGTGACCTC

c        A  R  P  E  T  P  S  P  A  I  P  S  H  S  S  N  T  A  L  E

         AGGCCTCTGGCTGTTCAGCTCGCGAGGAAAAGGACATCGTCGGAGGCCAGGCAGAAGCAG
   81009 -+---------+---------+---------+---------+---------+--------- 81068
         TCCGGAGACCGACAAGTCGAGCGCTCCTTTTCCTGTAGCAGCCTCCGGTCCGTCTTCGTC

c        R  P  L  A  V  Q  L  A  R  K  R  T  S  S  E  A  R  Q  K  G

         AAGCACCCCAAGAAAGTGAAGCAGGCCTTTAACCCCCTCATTTAACACCATGTTCTCGTG
```

- 3 -

```
81069 -+---------+---------+---------+---------+---------+--------- 81128
      TTCGTGGGGTTCTTTCACTTCGTCCGGAAATTGGGGGAGTAAATTGTGGTACAAGAGCAC

c     K  H  P  K  K  V  K  Q  A  E  N  P  L  I  *

      CAAGCAGCACCT
81129 -+----------+ 81140
      GTTCGTCGTGGA

c
```

B: p90



```
      AAGTGGTTCAGTGGACACCCACCACACAGCATGGCAACGACCAGTCATGTCGAGCATGAG
76377 ---+---------+---------+---------+---------+---------+------ 76436
      TTCACCAAGTCACCTGTGGGTGGTGTGTCGTACCGTTGCTGGTCAGTACAGCTCGTACTC

c                                           M  A  T  T  S  H  V  E  H  E

      CTCCTCTCCAAATTGATTGATGAGTTAAAGGTCAAGGCCAACTCAGACCCCGAGGCTGAT
76437 ---+---------+---------+---------+---------+---------+------ 76496
      GAGGAGAGGTTTAACTAACTACTCAATTTCCAGTTCCGGTTGAGTCTGGGGCTCCGACTA

c     L  L  S  K  L  I  D  E  L  K  V  K  A  N  S  D  P  E  A  D

      GTCCTGGCCGGGCGCCTGCTCCACCGCCTTAAGGCCGAGTCAGTTACACACACAGTAGCC
76497 ---+---------+---------+---------+---------+---------+------ 76556
      CAGGACCGGCCCGCGGACGAGGTGGCGGAATTCCGGCTCAGTCAATGTGTGTGTCATCGG

c     V  L  A  G  R  L  L  H  R  L  K  A  E  S  V  T  H  T  V  A

      GAATATCTGGAGGTCTTCTCTGACAAATTCTACGATGAGGAATTCTTCCAGATGCACCGG
76557 ---+---------+---------+---------+---------+---------+------ 76616
      CTTATAGACCTCCAGAAGAGACTGTTTAAGATGCTACTCCTTAAGAAGGTCTACGTGGCC

c     E  Y  L  E  V  F  S  D  K  F  Y  D  E  E  F  F  Q  M  H  R

      GATGAGCTGGAGACCCGAGTCTCTGCTTTCGCAGAGCCCGGCCTACGAGCGCATCGTC
76617 ---+---------+---------+---------+---------+---------+------ 76676
      CTACTCGACCTCTGGGCTCAGAGACGAAAGCGTCTCGGGCCGGATGCTCGCGTAGCAG

c     D  E  L  E  T  R  V  S  A  F  A  Q  S  P  A  Y  E  R  I  V

      TCCAGCGGCTACCTGTCGGCCCTGCGCTACTATGACACCTATCTGTATGTGGGGCGCAGC
76677 ---+---------+---------+---------+---------+---------+------ 76736
      AGGTCGCCGATGGACAGCCGGGACGCGATGATACTGTGGATAGACATACACCCCGCGTCG

c     S  S  G  Y  L  S  A  L  R  Y  Y  D  T  Y  L  Y  V  G  R  S

      GGGAAGCAGGAGAGTGTGCAGCACTTTTACATGCGGTTAGCCGGCTTCTGTGCCTCAACC
76737 ---+---------+---------+---------+---------+---------+------ 76796
      CCCTTCGTCCTCTCACACGTCGTGAAAATGTACGCCAATCGGCCGAAGACACGGAGTTGG

c     G  K  Q  E  S  V  Q  H  F  Y  M  R  L  A  G  F  C  A  S  T

      ACCTGCCTCTACGCGGGTCTCAGGGCAGCCCTGCAGCGGGCCAGGCCGGAGATTGAGAGT
76797 ---+---------+---------+---------+---------+---------+------ 76856
      TGGACGGAGATGCGCCCAGAGTCCCGTCGGGACGTCGCCCGGTCCGGCCTCTAACTCTCA

c     T  C  L  Y  A  G  L  R  A  A  L  Q  R  A  R  P  E  I  E  S

      GACATGGAGGTGTTTGATTACTACTTTGAGCACCTAACCTCCCAGACGGTGTGCTGCTCC
76857 ---+---------+---------+---------+---------+---------+------ 76916
      CTGTACCTCCACAAACTAATGATGAAACTCGTGGATTGGAGGGTCTGCCACACGACGAGG

c     D  M  E  V  F  D  Y  Y  F  E  H  L  T  S  Q  T  V  C  C  S

      ACGCCCTTTATGCGCTTTGCCGGGGTGGAAAACTCCACTCTGGCCAGCTGCATCCTCACC
76917 ---+---------+---------+---------+---------+---------+------ 76976
      TGCGGGAAATACGCGAAACGGCCCCACCTTTTGAGGTGAGACCGGTCGACGTAGGAGTGG
```

- 5 -

```
c       T  P  E  M  R  E  A  G  V  E  N  S  T  L  A  S  C  I  L  T

        ACCCCCGACCTCAGCTCCGAGTGGGACGTGACCCAGGCCCTCTATAGGCACCTGGGGCGC
  76977 ---+---------+---------+---------+---------+---------+------ 77036
        TGGGGGCTGGAGTCGAGGCTCACCCTGCACTGGGTCCGGGAGATATCCGTGGACCCCGCG

c       T  P  D  L  S  S  E  W  D  V  T  Q  A  L  Y  R  H  L  G  R

        TACCTCTTTCAGCGAGCCGGGGTGGGTGTAGGGGTGACGGGGGCTGGCCAGGATGGGAAA
  77037 ---+---------+---------+---------+---------+---------+------ 77096
        ATGGAGAAAGTCGCTCGGCCCCACCCACATCCCCACTGCCCCCGACCGGTCCTACCCTTT

c       Y  L  E  Q  R  A  G  V  G  V  G  V  T  G  A  G  Q  D  G  K

        CACATCAGCCTCCTGATGAGGATGATCAACAGCCACGTGGAGTACCACAACTATGGCTGC
  77097 ---+---------+---------+---------+---------+---------+------ 77156
        GTGTAGTCGGAGGACTACTCCTACTAGTTGTCGGTGCACCTCATGGTGTTGATACCGACG

c       H  I  S  L  L  M  R  M  I  N  S  H  V  E  Y  H  N  Y  G  C

        AAGAGGCCGGTCAGTGTGGCGGCCTACATGGAGCCCTGGCACAGCCAGATTTTCAAGTTT
  77157 ---+---------+---------+---------+---------+---------+------ 77216
        TTCTCCGGCCAGTCACACCGCCGGATGTACCTCGGGACCGTGTCGGTCTAAAAGTTCAAA

c       K  R  P  V  S  V  A  A  Y  M  E  P  W  H  S  Q  I  F  K  F

        TTGGAAACGAAGCTGCCGGAGAACCACGAGAGGTGCCCGGGCATCTTTACGGGGCTCTTT
  77217 ---+---------+---------+---------+---------+---------+------ 77276
        AACCTTTGCTTCGACGGCCTCTTGGTGCTCTCCACGGGCCCGTAGAAATGCCCCGAGAAA

c       L  E  T  K  L  P  E  N  H  E  R  C  P  G  I  F  T  G  L  F

        GTCCCCGAGCTCTTCTTCAAGCTTTTTAGGGACACGCCCTGGTCGGACTGGTACCTGTTT
  77277 ---+---------+---------+---------+---------+---------+------ 77336
        CAGGGGCTCGAGAAGAAGTTCGAAAAATCCCTGTGCGGGACCAGCCTGACCATGGACAAA

c       V  P  E  L  F  F  K  L  F  R  D  T  P  W  S  D  W  Y  L  F

        GACCCCAAGGACGCCGGGGACCTGGAGAGGCTCTACGGGGAGGAGTTTGAGCGCGAGTAC
  77337 ---+---------+---------+---------+---------+---------+------ 77396
        CTGGGGTTCCTGCGGCCCCTGGACCTCTCCGAGATGCCCCTCCTCAAACTCGCGCTCATG

c       D  P  K  D  A  G  D  L  E  R  L  Y  G  E  E  F  E  R  E  Y

        TATCGGCTGGTGACAGCGGGCAAGTTTTGTGGGCGGGTCTCCATCAAGTCCCTGATGTTC
  77397 ---+---------+---------+---------+---------+---------+------ 77456
        ATAGCCGACCACTGTCGCCCGTTCAAAACACCCGCCCAGAGGTAGTTCAGGGACTACAAG

c       Y  R  L  V  T  A  G  K  F  C  G  R  V  S  I  K  S  L  M  F

        TCTATCGTCAACTGCGCCGTCAAGGCCGGCAGCCCCTTCATCCTTTTGAAGGAGGCCTGC
  77457 ---+---------+---------+---------+---------+---------+------ 77516
        AGATAGCAGTTGACGCGGCAGTTCCGGCCGTCGGGGAAGTAGGAAAACTTCCTCCGGACG

c       S  I  V  N  C  A  V  K  A  G  S  P  F  I  L  L  K  E  A  C

        AACGCCCACTTTTGGCGCGACCTGCAGGGCGAGGCCATGAACGCCGCCAACCTGTGCGCC
  77517 ---+---------+---------+---------+---------+---------+------ 77576
        TTGCGGGTGAAAACCGCGCTGGACGTCCCGCTCCGGTACTTGCGGCGGTTGGACACGCGG

c       N  A  H  F  W  R  D  L  Q  G  E  A  M  N  A  A  N  L  C  A

        GAGGTGCTGCAGCCCTCGAGGAAGTCTGTGGCCACCTGCAATCTGGCCAACATCTGCCTC
```

```
77577 ---+---------+---------+---------+---------+---------+------ 77636
      CTCCACGACGTCGGGAGCTCCTTCAGACACCGGTGGACGTTAGACCGGTTGTAGACGGAG

c     E  V  L  Q  P  S  R  K  S  V  A  T  C  N  L  A  N  I  C  L

      CCGCGCTGCCTGGTGAATGCGCCTCTGGCGGTGCGGGCACAGCGGGCCGACACGCAGGGG
77637 ---+---------+---------+---------+---------+---------+------ 77696
      GGCGCGACGGACCACTTACGCGGAGACCGCCACGCCCGTGTCGCCCGGCTGTGCGTCCCC

c     P  R  C  L  V  N  A  P  L  A  V  R  A  Q  R  A  D  T  Q  G

      GATGAACTCCTGCTGGCCCTCCCTCGACTCTCAGTCACCCTACCTGGAGAGGGGGCAGTC
77697 ---+---------+---------+---------+---------+---------+------ 77756
      CTACTTGAGGACGACCGGGAGGGAGCTGAGAGTCAGTGGGATGGACCTCTCCCCCGTCAG

c     D  E  L  L  L  A  L  P  R  L  S  V  T  L  P  G  E  G  A  V

      GGTGATGGATTCTCGCTAGCCCGCCTCAGAGATGCCACCCAGTGTGCCACCTTTGTGGTG
77757 ---+---------+---------+---------+---------+---------+------ 77816
      CCACTACCTAAGAGCGATCGGGCGGAGTCTCTACGGTGGGTCACACGGTGGAAACACCAC

c     G  D  G  F  S  L  A  R  L  R  D  A  T  Q  C  A  T  F  V  V

      GCCTGCTCCATTCTTCAGGGATCCCCCACTTATGATTCCAGGGATATGGCCTCCATGGGC
77817 ---+---------+---------+---------+---------+---------+------ 77876
      CGGACGAGGTAAGAAGTCCCTAGGGGGTGAATACTAAGGTCCCTATACCGGAGGTACCCG

c     A  C  S  I  L  Q  G  S  P  T  Y  D  S  R  D  M  A  S  M  G

      CTCGGGGTGCAGGGCCTGGCCGATGTCTTTGCGGACCTGGGCTGGCAGTACACTGACCCT
77877 ---+---------+---------+---------+---------+---------+------ 77936
      GAGCCCCACGTCCCGGACCGGCTACAGAAACGCCTGGACCCGACCGTCATGTGACTGGGA

c     L  G  V  Q  G  L  A  D  V  F  A  D  L  G  W  Q  Y  T  D  P

      CCCTCTCGCTCGTTAAACAAGGAAATATTCGAACATATGTACTTTACGGCCCTCTGCACC
77937 ---+---------+---------+---------+---------+---------+------ 77996
      GGGAGAGCGAGCAATTTGTTCCTTTATAAGCTTGTATACATGAAATGCCGGGAGACGTGG

c     P  S  R  S  L  N  K  E  I  F  E  H  M  Y  E  T  A  L  C  T

      AGTAGTCTGATTGGACTTCACACCAGGAAGATTTTTCCGGGTTTCAAACAGAGCAAGTAT
77997 ---+---------+---------+---------+---------+---------+------ 78056
      TCATCAGACTAACCTGAAGTGTGGTCCTTCTAAAAAGGCCCAAAGTTTGTCTCGTTCATA

c     S  S  L  I  G  L  H  T  R  K  I  F  P  G  E  K  Q  S  K  Y

      GCCGGGGGGTGGTTTCACTGGCACGATTGGGCAGGAACAGACCTTTCTATTCCCAGGGAA
78057 ---+---------+---------+---------+---------+---------+------ 78116
      CGGCCCCCCACCAAAGTGACCGTGCTAACCCGTCCTTGTCTGGAAAGATAAGGGTCCCTT

c     A  G  G  W  F  H  W  H  D  W  A  G  T  D  L  S  I  P  R  E

      ATTTGGTCTCGCCTCTCTGAACGCATTGTGAGGGATGGGCTTTTCAATTCACAGTTTATC
78117 ---+---------+---------+---------+---------+---------+------ 78176
      TAAACCAGAGCGGAGAGACTTGCGTAACACTCCCTACCCGAAAAGTTAAGTGTCAAATAG

c     I  W  S  R  L  S  E  R  I  V  R  D  G  L  F  N  S  Q  E  I

      GCCCTGATGCCCACCTCAGGCTGTGCCCAGGTGACGGGCTGTTCGGACGCCTTCTACCCC
78177 ---+---------+---------+---------+---------+---------+------ 78236
      CGGGACTACGGGTGGAGTCCGACACGGGTCCACTGCCCGACAAGCCTGCGGAAGATGGGG
```

```
c          A  L  M  P  T  S  G  C  A  Q  V  T  G  C  S  D  A  E  Y  P

           TTCTATGCCAATGCGTCCACCAAGGTCACCAACAAGGAGGAGGCCCTTAGGCCAAACCGG
     78237 ---+---------+---------+---------+---------+---------+------ 78296
           AAGATACGGTTACGCAGGTGGTTCCAGTGGTTGTTCCTCCTCCGGGAATCCGGTTTGGCC

c          E  Y  A  N  A  S  T  K  V  T  N  K  E  E  A  L  R  P  N  R

           TCTTTTTGGCGTCATGTGCGTCTGGATGACAGGGAAGCTTTGAATCTTGTCGGGGGCCGT
     78297 ---+---------+---------+---------+---------+---------+------ 78356
           AGAAAAACCGCAGTACACGCAGACCTACTGTCCCTTCGAAACTTAGAACAGCCCCCGGCA

c          S  E  W  R  H  V  R  L  D  D  R  E  A  L  N  L  V  G  G  R

           GTCTCCTGCCTCCCGGAGGCTCTGCGGCAGCGCTACCTGCGTTTCCAAACGGCCTTTGAT
     78357 ---+---------+---------+---------+---------+---------+------ 78416
           CAGAGGACGGAGGGCCTCCGAGACGCCGTCGCGATGGACGCAAAGGTTTGCCGGAAACTA

c          V  S  C  L  P  E  A  L  R  Q  R  Y  L  R  E  Q  T  A  F  D

           TACAACCAGGAGGACCTGATTCAGATGTCCCGGGACAGGGCCCCCTTTGTGGACCAGAGC
     78417 ---+---------+---------+---------+---------+---------+------ 78476
           ATGTTGGTCCTCCTGGACTAAGTCTACAGGGCCCTGTCCCGGGGGAAACACCTGGTCTCG

c          Y  N  Q  E  D  L  I  Q  M  S  R  D  R  A  P  F  V  D  Q  S

           CAATCTCACAGCCTGTTTTTGCGTGAGGAAGATGCCGCGCGGGCCAGCACGCTAGCCAAC
     78477 ---+---------+---------+---------+---------+---------+------ 78536
           GTTAGAGTGTCGGACAAAAACGCACTCCTTCTACGGCGCGCCCGGTCGTGCGATCGGTTG

c          Q  S  H  S  L  F  L  R  E  E  D  A  A  R  A  S  T  L  A  N

           CTACTGGTGCGCAGCTACGAGCTGGGCCTGAAGACTATCATGTACTATTGTCGCATTGAG
     78537 ---+---------+---------+---------+---------+---------+------ 78596
           GATGACCACGCGTCGATGCTCGACCCGGACTTCTGATAGTACATGATAACAGCGTAACTC

c          L  L  V  R  S  Y  E  L  G  L  K  T  I  M  Y  Y  C  R  I  E

           AAGGCCGCCGATCTGGGGGTGATGGAGTGTAAGGCCAGCGCGGCTCTGTCGGTGCCGCGG
     78597 ---+---------+---------+---------+---------+---------+------ 78656
           TTCCGGCGGCTAGACCCCCACTACCTCACATTCCGGTCGCGCCGAGACAGCCACGGCGCC

c          K  A  A  D  L  G  V  M  E  C  K  A  S  A  A  L  S  V  P  R

           GAGGAACAGAATGAGCGGAGTCCCGCTGAGCAGATGCCGCCTCGTCCCATGGAACCGGCG
     78657 ---+---------+---------+---------+---------+---------+------ 78716
           CTCCTTGTCTTACTCGCCTCAGGGCGACTCGTCTACGGCGGAGCAGGGTACCTTGGCCGC

c          E  E  Q  N  E  R  S  P  A  E  Q  M  P  P  R  P  M  E  P  A

           CAGGTTGCGGGGCCGGTTGACATCATGAGCAAGGGCCCAGGGGAGGGACCAGGTGGGTGG
     78717 ---+---------+---------+---------+---------+---------+------ 78776
           GTCCAACGCCCCGGCCAACTGTAGTACTCGTTCCCGGGTCCCCTCCCTGGTCCACCCACC

c          Q  V  A  G  P  V  D  I  M  S  K  G  P  G  E  G  P  G  G  W

           TGTGTGCCCGGGGGATTGGAAGTGTGCTATAAGTACCGTCAGCTCTTCTCAGAGGATGAT
     78777 ---+---------+---------+---------+---------+---------+------ 78836
           ACACACGGGCCCCCTAACCTTCACACGATATTCATGGCAGTCGAGAAGAGTCTCCTACTA

c          C  V  P  G  G  L  E  V  C  Y  K  Y  R  Q  L  F  S  E  D  D

           CTGTTGGAGACTGACGGTTTTACTGAACGAGCCTGTGAATCTTGCCAAIAAACGTTTATT
```

```
78837 ---+---------+---------+---------+---------+---------+------ 78896
      GACAACCTCTGACTGCCAAAATGACTTGCTCGGACACTTAGAACGGTTATTTGCAAATAA

c     L  L  E  T  D  G  E  T  E  R  A  C  E  S  C  Q  *

      GCCATGTCCAAGTTGTTG
78897 ---+---------+---- 78914
      CGGTACAGGTTCAACAAC

c
```

C: p143 

```
      TTAGAGACACTATCACGTGTAACTTGACGTGCAAGGATGGAAGAGAGGGGCAGGGAAACG
 1700 +---------+---------+---------+---------+---------+--------- 1759
      AATCTCTGTGATAGTGCACATTGAACTGCACGTTCCTACCTTCTCTCCCCGTCCCTTTGC

                                             M  E  E  R  G  R  E  T
b
      CAAATGCCGGTTGCCCGGTATGGGGGCCCGTTTATTATGGTAAGGCTCTTCGGGCAAGAT
 1760 +---------+---------+---------+---------+---------+--------- 1819
      GTTTACGGCCAACGGGCCATACCCCCGGGCAAATAATACCATTCCGAGAAGCCCGTTCTA

b     Q  M  P  V  A  R  Y  G  G  P  F  I  M  V  R  L  F  G  Q  D

      GGAGAGGCAAACATACAGGAGGAAAGGCTATATGAGCTACTCTCTGACCCACGCTCCGCG
 1820 +---------+---------+---------+---------+---------+--------- 1879
      CCTCTCCGTTTGTATGTCCTCCTTTCCGATATACTCGATGAGAGACTGGGTGCGAGGCGC

b     G  E  A  N  I  Q  E  E  R  L  Y  E  L  L  S  D  P  R  S  A

      CTCGGCCTAGACCCGGGGCCCCTGATTGCTGAGAACCTGCTGCTAGTGGCGCTGCGTGGC
 1880 +---------+---------+---------+---------+---------+--------- 1939
      GAGCCGGATCTGGGCCCCGGGGACTAACGACTCTTGGACGACGATCACCGCGACGCACCG

b     L  G  L  D  P  G  P  L  I  A  E  N  L  L  L  V  A  L  R  G

      ACCAACAACGATCCCAGGCCTCAGCGTCAGGAGAGGGCCAGAGAACTGGCCCTCGTTGGC
 1940 +---------+---------+---------+---------+---------+--------- 1999
      TGGTTGTTGCTAGGGTCCGGAGTCGCAGTCCTCTCCCGGTCTCTTGACCGGGAGCAACCG

b     T  N  N  D  P  R  P  Q  R  Q  E  R  A  R  E  L  A  L  V  G

      ATTCTACTAGGAAACGGCGAGCAGGGTGAACACTTGGGCACGGAGAGTGCCCTGGAGGCC
 2000 +---------+---------+---------+---------+---------+--------- 2059
      TAAGATGATCCTTTGCCGCTCGTCCCACTTGTGAACCCGTGCCTCTCACGGGACCTCCGG

b     I  L  L  G  N  G  E  Q  G  E  H  L  G  T  E  S  A  L  E  A

      TCAGGCAACAACTATGTGTATGCCTACGGACCAGACTGGATGGCAAGGCCTTCCACATGG
 2060 +---------+---------+---------+---------+---------+--------- 2119
      AGTCCGTTGTTGATACACATACGGATGCCTGGTCTGACCTACCGTTCCGGAAGGTGTACC

b     S  G  N  N  Y  V  Y  A  Y  G  P  D  W  M  A  R  P  S  T  W

      TCCGCGGAAATCCAGCAATTCCTGCGACTCCTGGGCGCCACGTACGTGCTTCGCGTGGAG
 2120 +---------+---------+---------+---------+---------+--------- 2179
      AGGCGCCTTTAGGTCGTTAAGGACGCTGAGGACCCGCGGTGCATGCACGAAGCGCACCTC

b     S  A  E  I  Q  Q  F  L  R  L  L  G  A  T  Y  V  L  R  V  E

      ATGGGCAGGCAGTTTGGCTTCGAGGTGCATAGAAGCCGGCCCTCCTTCCGTCAGTTCCAG
 2180 +---------+---------+---------+---------+---------+--------- 2239
      TACCCGTCCGTCAAACCGAAGCTCCACGTATCTTCGGCCGGGAGGAAGGCAGTCAAGGTC

b     M  G  R  Q  F  G  F  E  V  H  R  S  R  P  S  F  R  Q  F  Q

      GCCATCAATCACCTTGTCCTGTTTGACAACGCCCTTCGCAAGTACGATTCCGGCCAGGTG
 2240 +---------+---------+---------+---------+---------+--------- 2299
      CGGTAGTTAGTGGAACAGGACAAACTGTTGCGGGAAGCGTTCATGCTAAGGCCGGTCCAC
```

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b        A  I  N  H  L  V  L  E  D  N  A  L  R  K  Y  D  S  G  Q  V

         GCGGCGGGCTTCCAGAGGGCCCTTCTGGTGGCCGGGCCAGAGACCGCTGACACGAGGCCG
    2300 +---------+---------+---------+---------+---------+--------- 2359
         CGCCGCCCGAAGGTCTCCCGGGAAGACCACCGGCCCGGTCTCTGGCGACTGTGCTCCGGC

b        A  A  G  E  Q  R  A  L  L  V  A  G  P  E  T  A  D  T  R  P

         GACCTCCGCAAGCTGAATGAGTGGGTGTTTGGTGGCAGGGCTGCTGGTGGCAGACAGCTG
    2360 +---------+---------+---------+---------+---------+--------- 2419
         CTGGAGGCGTTCGACTTACTCACCCACAAACCACCGTCCCGACGACCACCGTCTGTCGAC

b        D  L  R  K  L  N  E  W  V  F  G  G  R  A  A  G  G  R  Q  L

         GCCGACGAGCTAAAGATCGTGTCCGCGCTGCGAGACACTTACTCGGGCCACTTGGTCCTT
    2420 +---------+---------+---------+---------+---------+--------- 2479
         CGGCTGCTCGATTTCTAGCACAGGCGCGACGCTCTGTGAATGAGCCCGGTGAACCAGGAA

b        A  D  E  L  K  I  V  S  A  L  R  D  T  Y  S  G  H  L  V  L

         CAGCCCACGGAGACCCTTGACACATGGAAGGTGTTGAGCAGGGACACACGAACCGCTCAT
    2480 +---------+---------+---------+---------+---------+--------- 2539
         GTCGGGTGCCTCTGGGAACTGTGTACCTTCCACAACTCGTCCCTGTGTGCTTGGCGAGTA

b        Q  P  T  E  T  L  D  T  W  K  V  L  S  R  D  T  R  T  A  H

         AGTTTGGAGCACGGATTCATTCATGCCGCGGGGACCATCCAGGCCAACTGCCCACAGCTG
    2540 +---------+---------+---------+---------+---------+--------- 2599
         TCAAACCTCGTGCCTAAGTAAGTACGGCGCCCCTGGTAGGTCCGGTTGACGGGTGTCGAC

b        S  L  E  H  G  F  I  H  A  A  G  T  I  Q  A  N  C  P  Q  L

         TTTATGAGACGCCAGCACCCCGGCCTCTTTCCCTTCGTTAATGCAATAGCATCATCGCTG
    2600 +---------+---------+---------+---------+---------+--------- 2659
         AAATACTCTGCGGTCGTGGGGCCGGAGAAAGGGAAGCAATTACGTTATCGTAGTAGCGAC

b        F  M  R  R  Q  H  P  G  L  F  P  F  V  N  A  I  A  S  S  L

         GGCTGGTACTACCAGACCGCCACCGGCCCCGGAGCAGATGCCAGGGCGGCGGCCCGGCGC
    2660 +---------+---------+---------+---------+---------+--------- 2719
         CCGACCATGATGGTCTGGCGGTGGCCGGGGCCTCGTCTACGGTCCCGCCGCCGGGCCGCG

b        G  W  Y  Y  Q  T  A  T  G  P  G  A  D  A  R  A  A  A  R  R

         CAACAGGCCTTTCAGACCAGGGCGGCGGCTGAATGCCATGCCAAAAGCGGGGTGCCGGTC
    2720 +---------+---------+---------+---------+---------+--------- 2779
         GTTGTCCGGAAAGTCTGGTCCCGCCGCCGACTTACGGTACGGTTTTCGCCCCACGGCCAG

b        Q  Q  A  F  Q  T  R  A  A  A  E  C  H  A  K  S  G  V  P  V

         GTGGCCGGCTTCTACAGGACCATCAACGCCACGCTCAAGGGAGGAGAGGGCCTACAGCCC
    2780 +---------+---------+---------+---------+---------+--------- 2839
         CACCGGCCGAAGATGTCCTGGTAGTTGCGGTGCGAGTTCCCTCCTCTCCCGGATGTCGGG

b        V  A  G  F  Y  R  T  I  N  A  T  L  K  G  G  E  G  L  Q  P

         ACTATGTTTAACGGGGAGCTGGGGGCCATCAAGCACCAGGCACTTGACACTGTGAGGTAT
    2840 +---------+---------+---------+---------+---------+--------- 2899
         TGATACAAATTGCCCCTCGACCCCCGGTAGTTCGTGGTCCGTGAACTGTGACACTCCATA

b        T  M  F  N  G  E  L  G  A  I  K  H  Q  A  L  D  T  V  R  Y

         GACTACGGCCACTATCTCATAATGTTGGGGCCATTCCAGCCATGGAGCGGACTGACGGCC
```

```
2900 +---------+---------+---------+---------+---------+--------- 2959
     CTGATGCCGGTGATAGAGTATTACAACCCCGGTAAGGTCGGTACCTCGCCTGACTGCCGG

b    D  Y  G  H  Y  L  I  M  L  G  P  E  Q  P  W  S  G  L  T  A

     CCTCCGTGCCCCTACGCCGAAAGTTCATGGGCACAGGCGGCCGTGCAGACGGCCCTCGAG
2960 +---------+---------+---------+---------+---------+--------- 3019
     GGAGGCACGGGGATGCGGCTTTCAAGTACCCGTGTCCGCCGGCACGTCTGCCGGGAGCTC

b    P  P  C  P  Y  A  E  S  S  W  A  Q  A  A  V  Q  T  A  L  E

     CTGTTCTCGGCCCTGTACCCGGCCCCGTGCATCTCGGGCTACGCGCGCCCCCCGGGCCCC
3020 +---------+---------+---------+---------+---------+--------- 3079
     GACAAGAGCCGGGACATGGGCCGGGGCACGTAGAGCCCGATGCGCGCGGGGGGCCCGGGG

b    L  E  S  A  L  Y  P  A  P  C  I  S  G  Y  A  R  P  P  G  P

     AGTGCTGTGATCGAGCATCTGGGGTCCCTAGTTCCAAAGGGGGGTCTGCTGTTGTTTCTG
3080 +---------+---------+---------+---------+---------+--------- 3139
     TCACGACACTAGCTCGTAGACCCCAGGGATCAAGGTTTCCCCCCAGACGACAACAAAGAC

b    S  A  V  I  E  H  L  G  S  L  V  P  K  G  G  L  L  L  E  L

     TCTCACCTACCGGATGATGTTAAGGACGGGCTCGGAGAAATGGGGCCGGCCAGGGCCACG
3140 +---------+---------+---------+---------+---------+--------- 3199
     AGAGTGGATGGCCTACTACAATTCCTGCCCGAGCCTCTTTACCCCGGCCGGTCCCGGTGC

b    S  H  L  P  D  D  V  K  D  G  L  G  E  M  G  P  A  R  A  T

     GGACCTGGAATGCAGCAGTTTGTCAGCAGCTACTTCCTCAACCCCGCCTGTTCCAACGTC
3200 +---------+---------+---------+---------+---------+--------- 3259
     CCTGGACCTTACGTCGTCAAACAGTCGTCGATGAAGGAGTTGGGGCGGACAAGGTTGCAG

b    G  P  G  M  Q  Q  E  V  S  S  Y  E  L  N  P  A  C  S  N  V

     TTCATTACAGTGAGGCAGCGAGGGGAGAAGATCAACGGCCGTACCGTCCTCCAAGCGCTC
3260 +---------+---------+---------+---------+---------+--------- 3319
     AAGTAATGTCACTCCGTCGCTCCCCTCTTCTAGTTGCCGGCATGGCAGGAGGTTCGCGAG

b    E  I  T  V  R  Q  R  G  E  K  I  N  G  R  T  V  L  Q  A  L

     GGACGCGCATGCGATATGGCAGGCTGCCAGCACTATGTGCTGGGCTCCACGGTTCCCCTC
3320 +---------+---------+---------+---------+---------+--------- 3379
     CCTGCGCGTACGCTATACCGTCCGACGGTCGTGATACACGACCCGAGGTGCCAAGGGGAG

b    G  R  A  C  D  M  A  G  C  Q  H  Y  V  L  G  S  T  V  P  L

     GGTGGACTCAACTTTGTCAACGACCTGGCGTCCCCGGTTTCCACCGCCGAGATGATGGAT
3380 +---------+---------+---------+---------+---------+--------- 3439
     CCACCTGAGTTGAAACAGTTGCTGGACCGCAGGGGCCAAAGGTGGCGGCTCTACTACCTA

b    G  G  L  N  E  V  N  D  L  A  S  P  V  S  T  A  E  M  M  D

     GATTTCTCTCCCTTCTTCACCGTGGAGTTTCCCCCGATTCAAGAGGAGGGCGCAAGTTCT
3440 +---------+---------+---------+---------+---------+--------- 3499
     CTAAAGAGAGGGAAGAAGTGGCACCTCAAAGGGGGCTAAGTTCTCCTCCCGCGTTCAAGA

b    D  E  S  P  E  E  T  V  E  E  P  P  I  Q  E  E  G  A  S  S

     CCGGTACCCTTAGATGTGGACGAGAGCATGGACATCTCTCCGTCTTACGAGTTGCCCTGG
3500 +---------+---------+---------+---------+---------+--------- 3559
     GGCCATGGGAATCTACACCTGCTCTCGTACCTGTAGAGAGGCAGAATGCTCAACGGGACC
```

```
b        P   V   P   L   D   V   D   E   S   M   D   I   S   P   S   Y   E   L   P   W

         CTCTCGCTGGAGTCATGCCTCACAAGCATCCTGTCACACCCCACCGTGGGAAGCAAGGAG
    3560 +---------+---------+---------+---------+---------+--------- 3619
         GAGAGCGACCTCAGTACGGAGTGTTCGTAGGACAGTGTGGGGTGGCACCCTTCGTTCCTC

b        L   S   L   E   S   C   L   T   S   I   L   S   H   P   T   V   G   S   K   E

         CACTTGGTCAGGCACACGGACAGGGTCAGCGGAGGACGCGTGGCACAGCAGCCCGGGGTA
    3620 +---------+---------+---------+---------+---------+--------- 3679
         GTGAACCAGTCCGTGTGCCTGTCCCAGTCGCCTCCTGCGCACCGTGTCGTCGGGCCCCAT

b        H   L   V   R   H   T   D   R   V   S   G   G   R   V   A   Q   Q   P   G   V

         GGTCCCCTGGACCTGCCGCTGGCGGACTACGCCTTCGTTGCCCACAGTCAGGTCTGGACC
    3680 +---------+---------+---------+---------+---------+--------- 3739
         CCAGGGGACCTGGACGGCGACCGCCTGATGCGGAAGCAACGGGTGTCAGTCCAGACCTGG

b        G   P   L   D   L   P   L   A   D   Y   A   F   V   A   H   S   Q   V   W   T

         AGGCCCGGTGGGGCTCCTCCCTTGCCCTATCGTACCTGGGATCGAATGACAGAGAAGCTG
    3740 +---------+---------+---------+---------+---------+--------- 3799
         TCCGGGCCACCCCGAGGAGGGAACGGGATAGCATGGACCCTAGCTTACTGTCTCTTCGAC

b        R   P   G   G   A   P   P   L   P   Y   R   T   W   D   R   M   T   E   K   L

         CTTGTCTCCGCAAAACCCGGCGGAGAGAACGTTAAGGTTTCAGGTACCGTGATTACATTG
    3800 +---------+---------+---------+---------+---------+--------- 3859
         GAACAGAGGCGTTTTGGGCCGCCTCTCTTGCAATTCCAAAGTCCATGGCACTAATGTAAC

b        L   V   S   A   K   P   G   G   E   N   V   K   V   S   G   T   V   I   T   L

         GGAGAACAGGGGTACAAAGTGTCGTTGGATCTGAGGGAGGGAACCAGGCTGGCAATGGCT
    3860 +---------+---------+---------+---------+---------+--------- 3919
         CCTCTTGTCCCCATGTTTCACAGCAACCTAGACTCCCTCCCTTGGTCCGACCGTTACCGA

b        G   E   Q   G   Y   K   V   S   L   D   L   R   E   G   T   R   L   A   M   A

         GAGGCGCTGCTGAACGCAGCATGTGCCCCAATCTTGGATCCGGAAGACGTCTTGCTCACC
    3920 +---------+---------+---------+---------+---------+--------- 3979
         CTCCGCGACGACTTGCGTCGTACACGGGGTTAGAACCTAGGCCTTCTGCAGAACGAGTGG

b        E   A   L   L   N   A   A   C   A   P   I   L   D   P   E   D   V   L   L   T

         CTGCATCTACACCTGGATCCGCGCCGGGCAGACAACTCGGCCGTGATGGAGGCTATGACG
    3980 +---------+---------+---------+---------+---------+--------- 4039
         GACGTAGATGTGGACCTAGGCGCGGCCCGTCTGTTGAGCCGGCACTACCTCCGATACTGC

b        L   H   L   H   L   D   P   R   R   A   D   N   S   A   V   M   E   A   M   T

         GCGGCGAGTGACTACGCGCGTGGCCTGGGCGTGAAGCTGACCTTTGGCTCGGCCTCCTGC
    4040 +---------+---------+---------+---------+---------+--------- 4099
         CGCCGCTCACTGATGCGCGCACCGGACCCGCACTTCGACTGGAAACCGAGCCGGAGGACG

b        A   A   S   D   Y   A   R   G   L   G   V   K   L   T   F   G   S   A   S   C

         CCCGAGACCGGCTCGTCCGCCTCCAACTTCATGACCGTGGTGGCCTCTGTCTCCGCCCCA
    4100 +---------+---------+---------+---------+---------+--------- 4159
         GGGCTCTGGCCGAGCAGGCGGAGGTTGAAGTACTGGCACCACCGGAGACAGAGGCGGGGT

b        P   E   T   G   S   S   A   S   N   F   M   T   V   V   A   S   V   S   A   P

         GGGGAATTCTCGGGTCCTCTGATCACGCCAGTGCTTCAGAAGACGGGCAGTCTCCTGATT
```

```
4160 +---------+---------+---------+---------+---------+--------- 4219
     CCCCTTAAGAGCCCAGGAGACTAGTGCGGTCACGAAGTCTTCTGCCCGTCAGAGGACTAA

b     G  E  E  S  G  P  L  I  T  P  V  L  Q  K  T  G  S  L  L  I

     GCGGTGCGTTGCGGGGATGGCAAGATCCAGGGAGGGTCGCTGTTTGAGCAGCTCTTTAGC
4220 +---------+---------+---------+---------+---------+--------- 4279
     CGCCACGCAACGCCCCTACCGTTCTAGGTCCCTCCCAGCGACAAACTCGTCGAGAAATCG

b     A  V  R  C  G  D  G  K  I  Q  G  G  S  L  F  E  Q  L  F  S

     GACGTGGCCACGACCCCACGGGCACCCGAGGCGTTGTCTCTGAAGAATCTCTTCCGGGCA
4280 +---------+---------+---------+---------+---------+--------- 4339
     CTGCACCGGTGCTGGGGTGCCCGTGGGCTCCGCAACAGAGACTTCTTAGAGAAGGCCCGT

b     D  V  A  T  T  P  R  A  P  E  A  L  S  L  K  N  L  F  R  A

     GTCCAGCAGCTGGTCAAGAGCGGCATCGTGCTGTCAGGGCATGACATCAGCGACGGGGGC
4340 +---------+---------+---------+---------+---------+--------- 4399
     CAGGTCGTCGACCAGTTCTCGCCGTAGCACGACAGTCCCGTACTGTAGTCGCTGCCCCCG

b     V  Q  Q  L  V  K  S  G  I  V  L  S  G  H  D  I  S  D  G  G

     CTGGTGACCTGCCTGGTGGAGATGGCCCTGGCCGGGCAGCGGGGAGTGACCATCACTATG
4400 +---------+---------+---------+---------+---------+--------- 4459
     GACCACTGGACGGACCACCTCTACCGGGACCGGCCCGTCGCCCCTCACTGGTAGTGATAC

b     L  V  T  C  L  V  E  M  A  L  A  G  Q  R  G  V  T  I  T  M

     CCGGTGGCCTCCGACTACCTCCCGGAGATGTTTGCAGAGCACCCCGGCCTGGTGTTTGAG
4460 +---------+---------+---------+---------+---------+--------- 4519
     GGCCACCGGAGGCTGATGGAGGGCCTCTACAAACGTCTCGTGGGGCCGGACCACAAACTC

b     P  V  A  S  D  Y  L  P  E  M  F  A  E  H  P  G  L  V  F  E

     GTGGAGGAGCGCAGCGTGGGTGAGGTGCTGCAGACCCTGCGCTCCATGAACATGTACCCG
4520 +---------+---------+---------+---------+---------+--------- 4579
     CACCTCCTCGCGTCGCACCCACTCCACGACGTCTGGGACGCGAGGTACTTGTACATGGGC

b     V  E  E  R  S  V  G  E  V  L  Q  T  L  R  S  M  N  M  Y  P

     GCAGTCCTCGGTCGAGTGGGCGAGCAAGGTCCAGATCAAATGTTTGAGGTGCAGCACGGC
4580 +---------+---------+---------+---------+---------+--------- 4639
     CGTCAGGAGCCAGCTCACCCGCTCGTTCCAGGTCTAGTTTACAAACTCCACGTCGTGCCG

b     A  V  L  G  R  V  G  E  Q  G  P  D  Q  M  F  E  V  Q  H  G

     CCAGAGACGGTGTTGCGCCAGTCGCTGCGCCTGCTGCTGGGAACCTGGTCATCCTTTGCC
4640 +---------+---------+---------+---------+---------+--------- 4699
     GGTCTCTGCCACAACGCGGTCAGCGACGCGGACGACGACCCTTGGACCAGTAGGAAACGG

b     P  E  T  V  L  R  Q  S  L  R  L  L  L  G  T  W  S  S  F  A

     AGCGAGCAGTACGAGTGCCTGCGACCAGATCGGATTAACCGGTCCATGCACGTGTCCGAC
4700 +---------+---------+---------+---------+---------+--------- 4759
     TCGCTCGTCATGCTCACGGACGCTGGTCTAGCCTAATTGGCCAGGTACGTGCACAGGCTG

b     S  E  Q  Y  E  C  L  R  P  D  R  I  N  R  S  M  H  V  S  D

     TACGGCTATAACGAAGCACTGGCAGTCTCCCCGTTGACAGGAAAGAATCTCAGCCCACGC
4760 +---------+---------+---------+---------+---------+--------- 4819
     ATGCCGATATTGCTTCGTGACCGTCAGAGGGGCAACTGTCCTTTCTTAGAGTCGGGTGCG
```

```
b         Y  G  Y  N  E  A  L  A  V  S  P  L  T  G  K  N  L  S  F  R

          CGGTTGGTGACAGAGCCTGACCCACGATGTCAGGTGGCCGTGCTATGCGCCCCGGGCACC
   4820   +---------+---------+---------+---------+---------+---------  4879
          GCCAACCACTGTCTCGGACTGGGTGCTACAGTCCACCGGCACGATACGCGGGGCCCGTGG

b         R  L  V  I  E  P  D  P  R  C  Q  V  A  V  L  C  A  P  G  T

          AGGGGCCATGAAAGCCTCCTGGCGGCCTTCACGAATGCCGGATGCCTGTGCCGACGGGTG
   4880   +---------+---------+---------+---------+---------+---------  4939
          TCCCCGGTACTTTCGGAGGACCGCCGGAAGTGCTTACGGCCTACGGACACGGCTGCCCAC

b         R  G  H  E  S  L  L  A  A  E  T  N  A  G  C  L  C  R  R  V

          TTCTTTCGCGAGGTTAGGGACAACACGTTCCTCGACAAGTACGTGGGTCTGGCCATCGGA
   4940   +---------+---------+---------+---------+---------+---------  4999
          AAGAAAGCGCTCCAATCCCTGTTGTGCAAGGAGCTGTTCATGCACCCAGACCGGTAGCCT

b         E  E  R  E  V  R  D  N  T  E  L  D  K  Y  V  G  L  A  I  G

          GGAGTTCATGGGGCCAGGGACTCTGCCCTGGCAGGCCGTGCCACCGTGGCGCTGATTAAT
   5000   +---------+---------+---------+---------+---------+---------  5059
          CCTCAAGTACCCCGGTCCCTGAGACGGGACCGTCCGGCACGGTGGCACCGCGACTAATTA

b         G  V  H  G  A  R  D  S  A  L  A  G  R  A  T  V  A  L  I  N

          CGTTTCCCCGCCCTGCGTGACGCTATTCTAAAGTTCCTCAACAGGCCAGATACGTTCTCG
   5060   +---------+---------+---------+---------+---------+---------  5119
          GCAAAGGGGCGGGACGCACTGCGATAAGATTTCAAGGAGTTGTCCGGTCTATGCAAGAGC

b         R  E  P  A  L  R  D  A  I  L  K  E  L  N  R  P  D  T  E  S

          GTGGCCTTGGGGGAGCTGGGGGTGCAAGTTTTGGCTGGCCTGGGGGCCGTGGGGTCAACA
   5120   +---------+---------+---------+---------+---------+---------  5179
          CACCGGAACCCCCTCGACCCCCACGTTCAAAACCGACCGGACCCCCGGCACCCCAGTTGT

b         V  A  L  G  E  L  G  V  Q  V  L  A  G  L  G  A  V  G  S  I

          GATAATCCACCCGCCCCTGGCGTGGAAGTTAATGTCCAGAGATCACCTCTGATTCTGGCC
   5180   +---------+---------+---------+---------+---------+---------  5239
          CTATTAGGTGGGCGGGGACCGCACCTTCAATTACAGGTCTCTAGTGGAGACTAAGACCGG

b         D  N  P  P  A  P  G  V  E  V  N  V  Q  R  S  P  L  I  L  A

          CCCAACGCCTCTGGCATGTTTGAGTCCCGCTGGCTGAACATTAGCATCCCGGCGACCACC
   5240   +---------+---------+---------+---------+---------+---------  5299
          GGGTTGCGGAGACCGTACAAACTCAGGGCGACCGACTTGTAATCGTAGGGCCGCTGGTGG

b         P  N  A  S  G  M  E  E  S  R  W  L  N  I  S  I  P  A  T  T

          AGCTCTGTCATGCTGCGTGGCCTCCGGGGCTGCGTCCTGCCTTGTTGGGTGCAAGGCTCG
   5300   +---------+---------+---------+---------+---------+---------  5359
          TCGAGACAGTACGACGCACCGGAGGCCCCGACGCAGGACGGAACAACCCACGTTCCGAGC

b         S  S  V  M  L  R  G  L  R  G  C  V  L  P  C  W  V  Q  G  S

          TGCCTGGGCCTGCAATTTACTAACCTCGGGATGCCATATGTTTTGCAGAATGCCCACCAG
   5360   +---------+---------+---------+---------+---------+---------  5419
          ACGGACCCGGACGTTAAATGATTGGAGCCCTACGGTATACAAAACGTCTTACGGGTGGTC

b         C  L  G  L  W  E  T  N  L  G  M  P  Y  V  L  Q  N  A  H  W

          ATCGCCTGCCACTTCCACAGCAATGGCACGGATGCCTGGCGCTTTGCTATGAATTATCCA
```

```
5420 +---------+---------+---------+---------+---------+--------- 5479
     TAGCGGACGGTGAAGGTGTCGTTACCGTGCCTACGGACCGCGAAACGATACTTAATAGGT

b     I  A  C  H  E  H  S  N  G  T  D  A  W  R  E  A  M  N  Y  P

     AGAAACCCCACGGAGCAGGGCAACATTGCAGGGCTCTGTTCACGCGATGGTCGTCATCTG
5480 +---------+---------+---------+---------+---------+--------- 5539
     TCTTTGGGGTGCCTCGTCCCGTTGTAACGTCCCGAGACAAGTGCGCTACCAGCAGTAGAC

b     R  N  P  T  E  Q  G  N  I  A  G  L  C  S  R  D  G  R  H  L

     GCTCTCCTGTGTGACCCCTCACTTTGTACAGACTTTTGGCAATGGGAGCACATTCCCCCC
5540 +---------+---------+---------+---------+---------+--------- 5599
     CGAGAGGACACACTGGGGAGTGAAACATGTCTGAAAACCGTTACCCTCGTGTAAGGGGGG

b     A  L  L  C  D  P  S  L  C  T  D  E  W  Q  W  E  H  I  P  P

     GCCTTTGGGCACCCCACGGGGTGCTCCCCCTGGACACTTATGTTTCAAGCAGCTCACCTA
5600 +---------+---------+---------+---------+---------+--------- 5659
     CGGAAACCCGTGGGGTGCCCCACGAGGGGGACCTGTGAATACAAAGTTCGTCGAGTGGAT

b     A  E  G  H  P  T  G  C  S  P  W  T  L  M  E  Q  A  A  H  L

     TGGTCACTCAGGCACGGTCGCCCCTCCGAGTGACCAGTCAC
5660 +---------+---------+---------+---------+ 5700
     ACCAGTGAGTCCGTGCCAGCGGGGAGGCTCACTGGTCAGTG

b     W  S  L  R  H  G  R  P  S  E  *
```

D: p150



```
      ATCCATCTTTATTGACAATTATCAAAAAACCACCTTATTTCCAAACTTTAATATTCTTCG
43300 +---------+---------+---------+---------+---------+--------- 43359
      TAGGTAGAAATAACTGTTAATAGTTTTTTGGTGGAATAAAGGTTTGAAATTATAAGAAGC

d                             *  *  E  V  V  K  N  G  E  K  L  I  R  R

      TACCGGCGCCACCTCTTCAATTATATAGTGTCCGTAATGGATGGGGGCGTGGGTCTGTTT
43360 +---------+---------+---------+---------+---------+--------- 43419
      ATGGCCGCGGTGGAGAAGTTAATATATCACAGGCATTACCTACCCCCGCACCCAGACAAA

d     V  P  A  V  E  E  I  I  Y  H  G  Y  H  I  P  A  H  T  Q  K

      GACAGACATAAACTCATCGATGAGTGCCCGGGAGGAGGCTGAGAGTGCGGGGAATGCCTC
43420 +---------+---------+---------+---------+---------+--------- 43479
      CTGTCTGTATTTGAGTAGCTACTCACGGGCCCTCCTCCGACTCTCACGCCCCTTACGGAG

d     V  S  M  E  E  D  I  L  A  R  S  S  A  S  L  A  P  E  A  E

      CTGCAGAAAGCTGCAGGGCTGCTCCAGAAACACGTCAGTGCCAGCAATCACTACAAACTG
43480 +---------+---------+---------+---------+---------+--------- 43539
      GACGTCTTTCGACGTCCCGACGAGGTCTTTGTGCAGTCACGGTCGTTAGTGATGTTTGAC

d     Q  L  E  S  C  P  Q  E  L  E  V  D  T  G  A  I  V  V  E  Q

      CACCTCTGTGTTGCTGGTGGCTGGGTGCCCTCCAAGTCGCTGGCTGTACTCGTTGACCAT
43540 +---------+---------+---------+---------+---------+--------- 43599
      GTGGAGACACAACGACCACCGACCCACGGGAGGTTCAGCGACCGACATGAGCAACTGGTA

d     V  E  T  N  S  T  A  P  H  G  G  L  R  Q  S  Y  E  N  V  M

      GTTGTAGAGTCCCCTGTTGTTGCGCAGAAGCTCCTCCTTGTTGAAAAATGCCCGGCAGGG
43600 +---------+---------+---------+---------+---------+--------- 43659
      CAACATCTCAGGGGACAACAACGCGTCTTCGAGGAGGAACAACTTTTTACGGGCCGTCCC

d     N  Y  L  G  R  N  N  R  L  L  E  E  K  N  E  E  A  R  C  P

      GCTGTAGAGGCCCGGGACGGCCGTCTGGCGATAGGAGGAGTTGTACATGATGTCACCCAG
43660 ----------+---------+---------+---------+---------+--------- 43719
      CGACATCTCCGGGCCCTGCCGGCAGACCGCTATCCTCCTCAACATGTACTACAGTGGGTC

d     S  Y  L  G  P  V  A  T  Q  R  Y  S  S  N  Y  M  I  D  G  L

      AGAACCCAGCTGAGATGCCCAGGGATTCACAGTGCTCCGGTATTCATAGGCGGCATCCGG
43720 +---------+---------+---------+---------+---------+--------- 43779
      TCTTGGGTCGACTCTACGGGTCCCTAAGTGTCACGAGGCCATAAGTATCCGCCGTAGGCC

d     S  G  L  Q  S  A  W  P  N  V  T  S  R  Y  E  Y  A  A  D  P

      GCGAGAATGGTCATAGATGAGCCCCTCGGCAACCTCCTGATTGTAGTTTTCACAGGAGAC
43780 +---------+---------+---------+---------+---------+--------- 43839
      CGCTCTTACCAGTATCTACTCGGGGAGCCGTTGGAGGACTAACATCAAAAGTGTCCTCTG

d     R  S  H  D  Y  I  L  G  E  A  V  E  Q  N  Y  N  E  C  S  V

      CACACAGGCGGCCCGTCCCCTTGGAGAGTTGGACTTTTGAAAATAAGCCACGTCTGCCGT
43840 +---------+---------+---------+---------+---------+--------- 43899
      GTGTGTCCGCCGGGCAGGGGAACCTCTCAACCTGAAAACTTTTATTCGGTGCAGACGGCA
```

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d         V  C  A  A  R  G  R  P  S  N  S  K  Q  E  (  A  V  D  A  T

          GACCGGTGTTACGATAATCTCACAGGTGGCCTGCTGGCCGTGGCAGAGTCCTGGAGCTCC
   43900  +---------+---------+---------+---------+---------+--------- 43959
          CTGGCCACAATGCTATTAGAGTGTCCACCGGACGACCGGCACCGTCTCAGGACCTCGAGG

d         V  P  T  V  I  I  E  C  T  A  Q  Q  G  H  C  L  G  P  A  G

          ATTAACATTAGTCATACCTGCCAGGTATGTCCTGGGGTCCCGAAGCAGCGTCCCATTGCG
   43960  +---------+---------+---------+---------+---------+--------- 44019
          TAATTGTAATCAGTATGGACGGTCCATACAGGACCCCAGGGCTTCGTCGCAGGGTAACGC

d         N  V  N  T  M  G  A  L  Y  T  R  P  D  R  L  L  T  G  N  R

          CTGAGCGCCCACCTTGGCCTTGATGTAGTCATTGACTTGCTGGTTGCCAAAGGCCTCGGC
   44020  +---------+---------+---------+---------+---------+--------- 44079
          GACTCGCGGGTGGAACCGGAACTACATCAGTAACTGAACGACCAACGGTTTCCGGAGCCG

d         Q  A  G  V  K  A  K  I  Y  D  N  V  Q  Q  N  G  E  A  E  A

          CGGAAAGACGCTAAAGAAGTCTTGGGTGTGGATACCCATGTCAGTAGTGATGGCCGCCAC
   44080  +---------+---------+---------+---------+---------+--------- 44139
          GCCTTTCTGCGATTTCTTCAGAACCCACACCTATGGGTACAGTCATCACTACCGGCGGTG

d         P  E  V  S  E  E  D  Q  T  H  I  G  M  D  T  T  I  A  A  V

          CCTGGCCGGAGTCATGGTCGAGCTATAACTAAGCCCGGTGTCGATGGAGGCCATCTCGTG
   44140  +---------+---------+---------+---------+---------+--------- 44199
          GGACCGGCCTCAGTACCAGCTCGATATTGATTCGGGCCACAGCTACCTCCGGTAGAGCAC

d         R  A  P  T  M  T  S  S  Y  S  L  G  T  D  I  S  A  M  E  H

          ATGCACCTCAAAGGTTACCGCGTCCACCCTGGCCTCCCGGCGGCTAACATTTGGGGTCCC
   44200  +---------+---------+---------+---------+---------+--------- 44259
          TACGTGGAGTTTCCAATGGCGCAGGTGGGACCGGAGGGCCGCCGATTGTAAACCCCAGGG

d         H  V  E  E  T  V  A  D  V  R  A  E  R  R  S  V  N  P  T  G

          AATGAACATGGATGTTGAGGCCCTGGAGCTAAACAATATGTTTTCAGAGAGGATCTCATC
   44260  +---------+---------+---------+---------+---------+--------- 44319
          TTACTTGTACCTACAACTCCGGGACCTCGATTTGTTATACAAAAGTCTCTCCTAGAGTAG

d         I  E  m  S  T  E  A  R  S  S  E  L  I  N  E  S  L  I  E  D

          GGTCCTGACCACGGTCATGGCCACCCCTGGGTGGATCTTGAGCTTGGCCTGGGCAATATA
   44320  +---------+---------+---------+---------+---------+--------- 44379
          CCAGGACTGGTGCCAGTACCGGTGGGGACCCACCTAGAACTCGAACCGGACCCGTTATAT

d         T  R  V  V  T  M  A  V  G  P  H  I  K  L  K  A  Q  A  I  I

          GGCCATGGGGGACATCTTGATGTGCATGGCGGTCATTCCACTGATTGAAACGAGGGAAGG
   44380  +---------+---------+---------+---------+---------+--------- 44439
          CCGGTACCCCCTGTAGAACTACACGTACCGCCAGTAAGGTGACTAACTTTGCTCCCTTCC

d         A  M  P  S  M  K  I  H  M  A  T  M  G  S  I  S  V  L  S  P

          AAGACATTCGGCCGCGTATTTGCCCATGGGCGAGCGGTGCCACTCCCGGTACTCTGCAAA
   44440  +---------+---------+---------+---------+---------+--------- 44499
          TTCTGTAAGCCGGCGCATAAACGGGTACCCGCTCGCCACGGTGAGGGCCATGAGACGTTT

d         L  C  G  A  A  I  K  G  M  P  S  R  H  W  E  R  ,  E  A  F

          GAGCTGCTCTGGCCGGTTGAAGGCTTCCACGGCCCGCTGCTGAGGATTGCGCATAACAAA
```

```
44500 +---------+---------+---------+---------+---------+--------- 44559
      CTCGACGAGACCGGCCAACTTCCGAAGGTGCCGGGCGACGACTCCTAACGCGTATTGTTT

d     L  Q  E  P  R  N  E  A  E  V  A  R  Q  Q  P  N  R  M  V  E

      GGTGGCAACATCCTGGTGCATGGTGGCAGCCACTCGCGGGTCCCCGTAAAACATATGGAA
44560 +---------+---------+---------+---------+---------+--------- 44619
      CCACCGTTGTAGGACCACGTACCACCGTCGGTGAGCGCCCAGGGGCATTTTGTATACCTT

d     T  A  V  D  Q  H  M  T  A  A  V  R  P  D  G  Y  E  M  H  E

      AGGAATGGCGTGAAAGAGACACTGGGTGACGGCCCGGGTCCTCTCGGAGAAGGCAAAGGC
44620 +---------+---------+---------+---------+---------+--------- 44679
      TCCTTACCGCACTTTCTCTGTGACCCACTGCCGGGCCCAGGAGAGCCTCTTCCGTTTCCG

d     P  I  A  H  E  L  C  Q  T  V  A  R  T  R  E  S  E  A  E  A

      CACCAGCCCGTTCACCAAAACAGTCTGCTCTGTCCGCTTGTCGGCGGGATTCGGGGCCAG
44680 +---------+---------+---------+---------+---------+--------- 44739
      GTGGTCGGGCAAGTGGTTTTGTCAGACGAGACAGGCGAACAGCCGCCCTAAGCCCCGGTC

d     V  L  G  N  V  L  V  T  Q  E  T  R  K  D  A  P  N  P  A  L

      CTGCTGCGTAACGTCATTGTCCACCGACACACGCACGGCACGGGTGAAAGTGGGGCAGGT
44740 +---------+---------+---------+---------+---------+--------- 44799
      GACGACGCATTGCAGTAACAGGTGGCTGTGTGCGTGCCGTGCCCACTTTCACCCCGTCCA

d     Q  Q  T  V  D  N  D  V  S  V  R  V  A  R  T  E  T  P  C  T

      CATGAATGAGGCGCTGAGGTCCCTGATCATGCCCACGGTGGGGCGGAGGTCGGAGATCTC
44800 +---------+---------+---------+---------+---------+--------- 44859
      GTACTTACTCCGCGACTCCAGGGACTAGTACGGGTGCCACCCCGCCTCCAGCCTCTAGAG

d     M  E  S  A  S  L  D  R  I  M  G  V  T  P  R  L  D  S  I  E

      CAGCAGATCCCTGAGCGTCCCATTCTCCAAATTGTCGAGGATGTCCTCGTCCCTGGTAAA
44860 +---------+---------+---------+---------+---------+--------- 44919
      GTCGTCTAGGGACTCGCAGGGTAAGAGGTTTAACAGCTCCTACAGGAGCAGGGACCATTT

d     L  L  D  R  L  T  G  N  E  L  N  D  L  I  D  E  D  R  T  E

      ATGGTGGCTGAAGGCTGGCCCGTTGTAGGCCAGGGTCTGGGCCACGTGCTGAAAGTCCAC
44920 +---------+---------+---------+---------+---------+--------- 44979
      TACCACCGACTTCCGACCGGGCAACATCCGGTCCCAGACCCGGTGCACGACTTTCAGGTG

d     H  H  S  E  A  P  G  N  Y  A  L  T  Q  A  V  H  Q  E  D  V

      CCCGAGGCCGCACATGTGGGCATTGGTGCAGGTTGGGAGGAAAACGTAGTAAAAGATCTT
44980 +---------+---------+---------+---------+---------+--------- 45039
      GGGCTCCGGCGTGTACACCCGTAACCACGTCCAACCCTCCTTTTGCATCATTTTCTAGAA

d     G  L  G  C  M  H  A  N  T  C  T  P  L  E  V  Y  Y  E  I  K

      TTCCAGCACATCCGCATGCCCCTCATCTACATAAGGGCCTAGGTGCAGACGGAAATCGTG
45040 +---------+---------+---------+---------+---------+--------- 45099
      AAGGTCGTGTAGGCGTACGGGGAGTAGATGTATTCCCGGATCCACGTCTGCCTTTAGCAC

d     E  L  V  D  A  H  G  E  D  V  Y  P  G  L  H  L  R  E  D  H

      GTCGTGGTCTCCGTTAACCCGGTAGCCGTACAAGGCCACAAATTGGGCAGCCATCTCATC
45100 +---------+---------+---------+---------+---------+--------- 45159
      CAGCACCAGAGGCAATTGGGCCATCGGCATGTTCCGGTGTTTAACCCGTCGGTAGAGTAG
```

```
d            D  H  D  G  N  V  R  Y  G  Y  L  A  V  E  Q  A  A  M  E  D

             CATGTTTCCAACCCTCTCAATAAACTGGGGCGCGGCCAGGGTGTCAGCGTAAACCTCATT
      45160  +---------+---------+---------+---------+---------+---------  45219
             GTACAAAGGTTGGGAGAGTTATTTGACCCCGCGCCGGTCCCACAGTCGCATTTGGAGTAA

d            M  N  G  V  R  E  I  E  Q  P  A  A  L  T  D  A  Y  V  E  N

             TCCGATAATAATCTGGGGGGCCCGGTCACTAACGGTGAGAAGATGGGTGAAAATGTCTGT
      45220  +---------+---------+---------+---------+---------+---------  45279
             AGGCTATTATTAGACCCCCCGGGCCAGTGATTGCCACTCTTCTACCCACTTTTACAGACA

d            G  I  I  I  Q  P  A  R  D  S  V  T  L  L  H  T  E  I  D  T

             GTAGGCCACCGGGGGGAGCAGGTTAGGGTCCAGGAGAGCGCAGACATACTGACCCACGCT
      45280  +---------+---------+---------+---------+---------+---------  45339
             CATCCGGTGGCCCCCCTCGTCCAATCCCAGGTCCTCTCGCGTCTGTATGACTGGGTGCGA

d            Y  A  V  P  P  L  L  N  P  D  L  L  A  C  V  Y  Q  G  V  S

             CTCATCCCCCACAACATCTGACCCGGCCAGGCGCATCAGGGCCTGCTCTAGGGCTATAAG
      45340  +---------+---------+---------+---------+---------+---------  45399
             GAGTAGGGGGTGTTGTAGACTGGGCCGGTCCGCGTAGTCCCGGACGAGATCCCGATATTC

d            E  D  G  V  V  D  S  G  A  L  R  M  L  A  Q  E  L  A  I  L

             TTCCCCATAGATTTTTCTATACATGGAATAGGCCTCCTTGGAGATGGCGTTATTTCCCAG
      45400  +---------+---------+---------+---------+---------+---------  45459
             AAGGGGTATCTAAAAAGATATGTACCTTATCCGGAGGAACCTCTACCGCAATAAAGGGTC

d            E  G  (  I  K  R  Y  M  S  Y  A  E  K  S  I  A  N  N  G  L

             GTGGCGGCAGATGAACTTGATCATGGAAAAGCTGTTCACAAAGGCAAGCCTCCCTGACCG
      45460  +---------+---------+---------+---------+---------+---------  45519
             CACCGCCGTCTACTTGAACTAGTACCTTTTCGACAAGTGTTTCCGTTCGGAGGGACTGGC

d            H  R  C  I  E  K  I  M  S  E  S  N  V  E  A  L  R  G  S  R

             TTCCCAGTAGGTGTTGATGCACAGGGACACCAAAGGCACGTTCATGACAAACTTTTCCTC
      45520  +---------+---------+---------+---------+---------+---------  45579
             AAGGGTCATCCACAACTACGTGTCCCTGTGGTTTCCGTGCAAGTACTGTTTGAAAAGGAG

d            E  W  Y  T  N  I  C  L  S  V  L  P  V  N  M  V  E  K  E  E

             AAACCCGTGGATCATAGCCTCGACTACGTAGAAGAAGGCTGGATAGGCAGTGTCATAGGC
      45580  +---------+---------+---------+---------+---------+---------  45639
             TTTGGGCACCTAGTATCGGAGCTGATGCATCTTCTTCCGACCTATCCGTCACAGTATCCG

d            E  G  H  I  M  A  E  V  V  Y  E  E  A  P  Y  A  T  D  Y  A

             AGTATCCTGCACAGTCTCAATAACGGCCTGATCCACCACGTGGGCCAGAGATGTGGCGGT
      45640  +---------+---------+---------+---------+---------+---------  45699
             TCATAGGACGTGTCAGAGTTATTGCCGGACTAGGTGGTGCACCCGGTCTCTACACCGCCA

d            T  D  Q  V  T  E  I  V  A  Q  D  V  V  H  A  L  S  T  A  T

             CTCAAACTGCTGCCCCCGGGCCTCTTGGAATGCAGCTGGGGCCAGGGGAGTCGGCAGGTT
      45700  +---------+---------+---------+---------+---------+---------  45759
             GAGTTTGACGACGGGGGCCCGGAGAACCTTACGTCGACCCCGGTCCCCTCAGCCGTCCAA

d            S  E  Q  Q  G  R  A  E  Q  E  A  A  P  A  L  P  T  P  L  N

             ACCCACCATTAGCCGGTGCACAGCCCTGTGCCTGGCCCTCTCCCCGGCATCCCTGCCAAT
```

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45760 +---------+---------+---------+---------+---------+--------- 45819
      TGGGTGGTAATCGGCCACGTGTCGGGACACGGACCGGGAGAGGGGCCGTAGGGACGGTTA

d      G  V  M  L  R  H  V  A  R  H  R  A  R  E  G  A  D  R  G  I

      GTAAATATCATAAAGGGGGTGCAGCTCCAGCCGCAGCAGGTCATAATTGGACGGGTGGAG
45820 +---------+---------+---------+---------+---------+--------- 45879
      CATTTATAGTATTTCCCCCACGTCGAGGTCGGCGTCGTCCAGTATTAACCTGCCCACCTC

d      Y  I  D  Y  L  P  H  L  E  L  R  L  L  D  Y  N  S  P  H  L

      GAAGTCTTCGGTGGGCAGCCCGCACTTGAGAGCTATATCTGTCACGGGGGCTGCATACTT
45880 +---------+---------+---------+---------+---------+--------- 45939
      CTTCAGAAGCCACCCGTCGGGCGTGAACTCTCGATATAGACAGTGCCCCCGACGTATGAA

d      E  D  E  T  P  L  G  C  K  L  A  I  D  I  V  P  A  A  Y  K

      GTTATCATAGAACTCGTCCACAATAACAAGCACATTCATGTGATTGGGCCTCCTGTGTTG
45940 +---------+---------+---------+---------+---------+--------- 45999
      CAATAGTATCTTGAGCAGGTGTTATTGTTCGTGTAAGTACACTAACCCGGAGGACACAAC

d      N  D  Y  E  E  D  V  I  V  L  V  N  M  H  N  P  R  R  H  Q

      CAGGGAGTAGGTCTCGCGCCTGTCTCGCGGGGCCGGGGCCGCGTTGAGGCTGTTTAGGGT
46000 +---------+---------+---------+---------+---------+--------- 46059
      GTCCCTCATCCAGAGCGCGGACAGAGCGCCCCGGCCCCGGCGCAACTCCGACAAATCCCA

d      L  S  Y  T  E  R  R  D  R  P  A  P  A  A  N  L  S  N  L  T

      ATGGGCGGGTGTGTGGAGTCGGGGGTGACAGAGAACCTTGAGAGCATTCTGTAGGTTAAA
46060 +---------+---------+---------+---------+---------+--------- 46119
      TACCCGCCCACACACCTCAGCCCCCACTGTCTCTTGGAACTCTCGTAAGACATCCAATTT

d      H  A  P  T  H  L  R  P  H  C  L  V  K  L  A  N  Q  L  N  E

      CGCGAGGAGAAGGTTATTCTTGTTTACGATCCATGCCTCCACCGGTAGCTGCTGTGTGGG
46120 +---------+---------+---------+---------+---------+--------- 46179
      GCGCTCCTCTTCCAATAAGAACAAATGCTAGGTACGGAGGTGGCCATCGACGACACACCC

d      A  L  L  L  N  N  K  N  V  I  W  A  E  V  P  L  Q  Q  T  F

      GTTGTCCAGCATTTTGATGGCGGCGGAGGTCGTGTACTTGGGATTGGGCATAAACAGGCC
46180 +---------+---------+---------+---------+---------+--------- 46239
      CAACAGGTCGTAAAACTACCGCCGCCTCCAGCACATGAACCCTAACCCGTATTTGTCCGG

d      N  D  L  M  K  I  A  A  S  T  T  Y  K  P  N  P  M  E  L  G

      CACTGGGAAATAGTAGCTGTACTGCATTCTTCTGTTGAGGGGGTATGGGGACTGAGTGTC
46240 +---------+---------+---------+---------+---------+--------- 46299
      GTGACCCTTTATCATCGACATGACGTAAGAAGACAACTCCCCCATACCCCTGACTCACAG

d      V  P  E  Y  Y  S  Y  Q  M  R  R  N  L  P  Y  P  S  Q  T  D

      ATTGTACATCTTTTGCAGGCTTTCCACGGCCACCGCGTGGTTGCCCAGCTTGATGACGGC
46300 +---------+---------+---------+---------+---------+--------- 46359
      TAACATGTAGAAAACGTCCGAAAGGTGCCGGTGGCGCACCAACGGGTCGAACTACTGCCG

d      N  Y  M  K  Q  L  S  E  V  A  V  A  H  N  G  L  K  I  V  A

      GGCTGAGATCGGCACCCGGGGCTGATCCTCGACCCCTGCGGCCACAGCCGGCAGGTCAGA
46360 +---------+---------+---------+---------+---------+--------- 46419
      CCGACTCTAGCCGTGGGCCCCGACTAGGAGCTGGGGACGCCGGTGTCGGCCGTCCAGTCT
```

```
d        A  S  I  P  V  R  P  Q  D  E  V  G  A  A  V  A  P  L  D  S

         CTTGGTGCTTCCGGCTTTTTCCGGTGAGTCCACGATCCTAGCCATGAAATGCTCAAACGT
   46420 +---------+---------+---------+---------+---------+--------- 46479
         GAACCACGAAGGCCGAAAAAGGCCACTCAGGTGCTAGGATCGGTACTTTACGAGTTTGCA

d        K  T  S  G  A  K  E  P  S  D  V  I  R  A  M  E  H  E  E  T

         ACGCATCACGCGCCCGTAGCTCACGGCAGTGACCAGGTTCTCCCCCCGTACCACAAAAGA
   46480 +---------+---------+---------+---------+---------+--------- 46539
         TGCGTAGTGCGCGGGCATCGAGTGCCGTCACTGGTCCAAGAGGGGGGCATGGTGTTTTCT

d        R  M  V  R  G  Y  S  V  A  I  V  L  N  E  G  R  V  V  E  S

         AGCATAGCTCGAGGGCCCCATAATCTGGTTGTCGGCCTCCTCACCCAGGAAGGTCAAGAG
   46540 +---------+---------+---------+---------+---------+--------- 46599
         TCGTATCGAGCTCCCGGGGTATTAGACCAACAGCCGGAGGAGTGGGTCCTTCCAGTTCTC

d        A  Y  S  S  P  G  M  I  Q  N  D  A  E  E  G  L  E  T  L  L

         CTGGCGCAGAACGTTGTCGGTGACAATAAACACCCCCCCACTGGCTCTCCCCCCTTGGC
   46600 +---------+---------+---------+---------+---------+--------- 46659
         GACCGCGTCTTGCAACAGCCACTGTTATTTGTGGGGGGGTGACCGAGAGGGGGGAACCG

d        Q  R  L  V  N  D  T  V  I  E  V  G  G  V  P  E  G  G  K  A

         GGTCGTGTAGGTACTGACCCCCTTGAGCACGCTCTCCCCGGACACGGCCGCTACCATCTC
   46660 +---------+---------+---------+---------+---------+--------- 46719
         CCAGCACATCCATGACTGGGGGAACTCGTGCGAGAGGGGCCTGTGCCGGCGATGGTAGAG

d        T  T  Y  T  S  V  G  K  L  V  S  E  G  S  V  A  A  V  M  E

         AGAGAGACGGCTTCGCACGTACTGAGAAAACCCGGAGCCCATGTTCTCGGCCCGGTCCAG
   46720 +---------+---------+---------+---------+---------+--------- 46779
         TCTCTCTGCCGAAGCGTGCATGACTCTTTTGGGCCTCGGGTACAAGAGCCGGGCCAGGTC

d        S  L  R  S  R  V  Y  Q  S  E  G  S  G  M  N  E  A  R  D  L

         GAAGAAGGAGTGCTCCAGCAGATGCCTCTTGAACATGGCAATGAGGTCAGACTTGACAGT
   46780 +---------+---------+---------+---------+---------+--------- 46839
         CTTCTTCCTCACGAGGTCGTCTACGGAGAACTTGTACCGTTACTCCAGTCTGAACTGTCA

d        E  E  S  H  E  L  L  H  R  K  E  M  A  I  L  D  S  K  V  T

         CTTGGAGAACCCCCTCTCAGTGAAGGTGGGATCCGCCAGGGTCTGCAGGATAAACATGGG
   46840 +---------+---------+---------+---------+---------+--------- 46899
         GAACCTCTTGGGGGAGAGTCACTTCCACCCTAGGCGGTCCCAGACGTCCTATTTGTACCC

d        K  S  E  G  R  E  T  E  T  P  D  A  L  T  Q  L  I  S  M  P

         AGGGGCATGGCGAAGCTTCACACTCAGGACGGTGTTAATGAGGCCCCTCTCCAGGGCATC
   46900 +---------+---------+---------+---------+---------+--------- 46959
         TCCCCGTACCGCTTCGAAGTGTGAGTCCTGCCACAATTACTCCGGGGAGAGGTCCCGTAG

d        P  A  H  R  L  K  V  S  L  V  T  N  I  L  G  R  E  L  A  D

         GACCCCAAACTGTAGGGCCGAGGCCACGGTCTTGACAGCCCCCACGTACTCTGCGTACTC
   46960 +---------+---------+---------+---------+---------+--------- 47019
         CTGGGGTTTGACATCCCGGCTCCGGTGCCAGAACTGTCGGGGGTGCATGAGACGCATGAG

d        D  G  E  D  L  A  S  A  V  T  K  V  A  G  V  Y  E  A  [illegible]  E

         GACCGGGGTCTCGGGGATACTATGCAGGATCTCCAGATCCAGCATGGACAGTTCCATTTC
```

```
47020 +---------+---------+---------+---------+---------+--------- 47079
      CTGGCCCCAGAGCCCCTATGATACGTCCTAGAGGTCTAGGTCGTACCTGTCAAGGTAAAG

d     V  P  T  E  P  I  S  H  L  I  E  L  D  L  M  S  L  E  M  E

      CGTACTAATGTGGTGTTTGTGGCAATTTTTGACCACAATGAATGTCCGCTGCTTGCTGGG
47080 +---------+---------+---------+---------+---------+--------- 47139
      GCATGATTACACCACAAACACCGTTAAAAACTGGTGTTACTTACAGGCGACGAACGACCC

d     T  S  I  H  H  K  H  C  N  K  V  V  I  E  T  R  Q  K  S  P

      TCTCCTTCCGTCCCCGTGAGCAATGGTGGGGACGGAGATTCGAAATTGAATCTTGCCATC
47140 +---------+---------+---------+---------+---------+--------- 47199
      AGAGGAAGGCAGGGGCACTCGTTACCACCCCTGCCTCTAAGCTTTAACTTAGAACGGTAG

d     R  R  G  D  G  H  A  I  T  P  V  S  I  R  E  Q  I  K  G  D

      CGTCATACGACTCAGGTCTTTGAATTCCGTGTTCACACAGGACACGGCCAGTGCCGTCTC
47200 +---------+---------+---------+---------+---------+--------- 47259
      GCAGTATGCTGAGTCCAGAAACTTAAGGCACAAGTGTGTCCTGTGCCGGTCACGGCAGAG

d     T  M  R  S  L  D  K  E  E  T  N  V  C  S  V  A  L  A  T  E

      CAGGAAGCGAACATATTGGATGGCGTTCGTGTAGACCCCGAGTAGCACCTCAAACTTGAT
47260 +---------+---------+---------+---------+---------+--------- 47319
      GTCCTTCGCTTGTATAACCTACCGCAAGCACATCTGGGGCTCATCGTGGAGTTTGAACTA

d     L  E  R  V  Y  Q  I  A  N  T  Y  V  G  L  L  V  E  E  K  I

      GCCCGCCTCTCTGGCATCCTTGCCCACCAGCAGGTCAAAGCTATGAAACAACCCCTCAGC
47320 +---------+---------+---------+---------+---------+--------- 47379
      CGGGCGGAGAGACCGTAGGAACGGGTGGTCGTCCAGTTTCGATACTTTGTTGGGGAGTCG

d     G  A  E  R  A  D  K  G  V  L  L  D  E  S  H  E  L  G  E  A

      CGCTGACTGCCGCAGGTTCGAGAGCAGGTCGGCATCCACCGTCAGATAGGGGAAGGGTCT
47380 +---------+---------+---------+---------+---------+--------- 47439
      GCGACTGACGGCGTCCAAGCTCTCGTCCAGCCGTAGGTGGCAGTCTATCCCCTTCCCAGA

d     A  S  G  R  L  N  E  L  L  D  A  D  V  T  L  Y  P  E  P  R

      GTTTTCCACACCCTCATTTGAGGCCATGACACAAGGTAAGAGGGAGATGGGGGGAGG
47440 +---------+---------+---------+---------+---------+------ 47496
      CAAAAGGTGTGGGAGTAAACTCCGGTACTGTGTTCCATTCTCCCTCTACCCCCCTCC

d     N  S  V  G  E  N  S  A  M
```

Figure 29:
Expression of the ß-gal::p150 fusion proteins

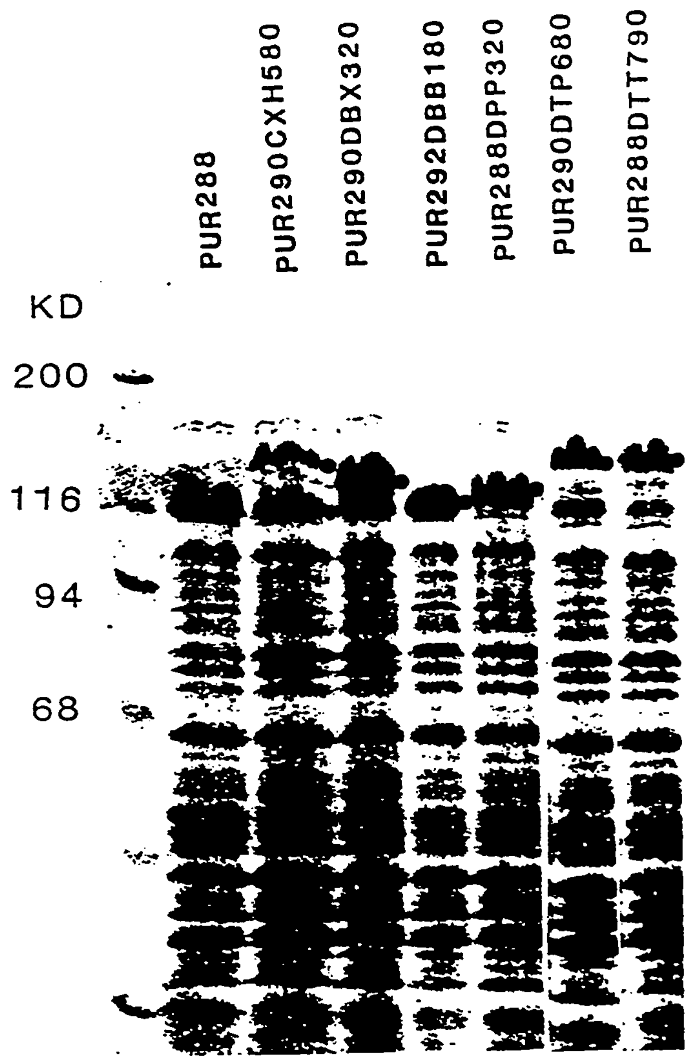


Figure 30:
Antigenicity of the ß-gal::p150 fusion proteins

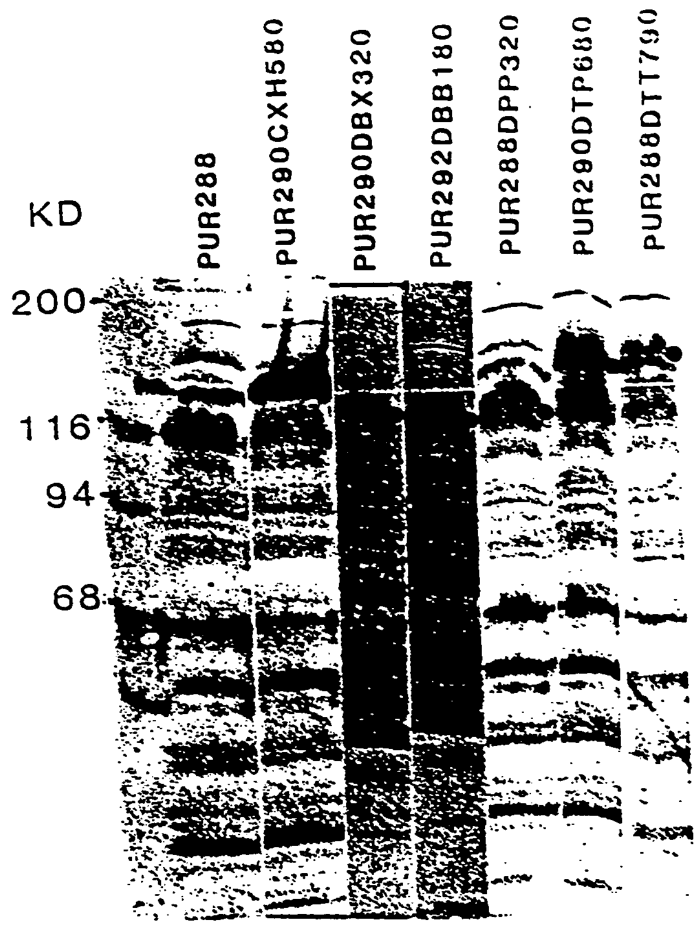
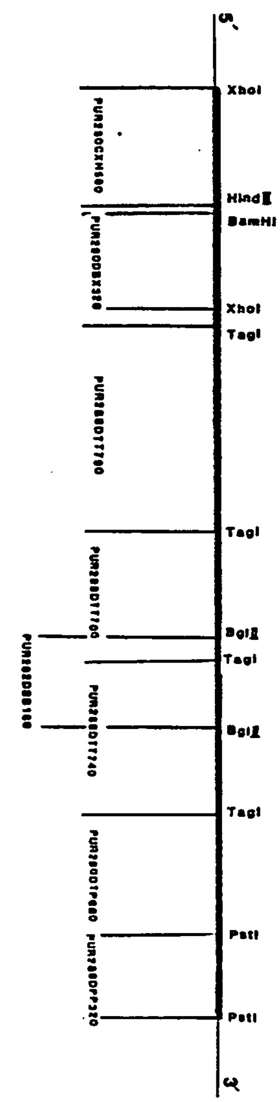


Figure 31:

Map of the p150 encoding region



0173254

European Patent Office

Application number:

85110565.0

# DECLARATION PURSUANT TO RULE 28, PARAGRAPH 4, OF THE EUROPEAN PATENT CONVENTION

The applicant has informed the European Patent Office that, until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, the availability of the micro-organism(s) identified below, referred to in paragraph 3 of Rule 28 of the European Patent Convention, shall be effected only by the issue of a sample to an expert.

## IDENTIFICATION OF THE MICRO-ORGANISMS

Accession numbers of the deposits:

pUCARG 680

DSM 3408

European Patent Office

# PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application number: EP 85110565.0

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. Cl.4) |
|---|---|---|---|
| P,X | EP - A2 - 0 151 079 (THE UNIVERSITY OF CHICAGO)<br>* Claims 1-6 *<br>-- | 1,12,<br>17,18,<br>24 | C 12 N 15/00<br>C 12 P 19/34<br>C 12 N 7/00<br>C 12 N 1/20<br>C 12 N 1/16<br>C 12 N 5/00<br>C 12 P 21/02<br>C 07 K 13/00<br>A 61 K 39/245<br>G 01 N 33/53<br>C 07 K 17/00<br>//C 12 R 1:19<br>C 12 R 1:91 |
| D,A | INTERNATIONAL JOURNAL OF CANCER, vol. 23, no. 6, June 15, 1979, Helsinki<br>L.F. QUALTIERE et al. "Epstein-Barr Virus-Induced Membrane Antigens: Immunochemical Characterisation of Triton X-100 Solubilized Viral Membrane Antigens from EBV-Superinfected Raji Cells"<br>pages 808-817<br>* Totality *<br>---- | 1,19 | |

| TECHNICAL FIELDS SEARCHED (Int. Cl.4) |
|---|
| C 12 N<br>C 12 P<br>C 07 K<br>A 61 K<br>G 01 N |

## INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely: –
Claims searched incompletely: –
Claims not searched: Claim 25
Reason for the limitation of the search:

(Article 52(4) EPC)

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| VIENNA | 19-11-1985 | WOLF |

**CATEGORY OF CITED DOCUMENTS**

X : particularly relevant if taken alone
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